RNA 3' CLEAVAGE AND POLYADENYLATION IN OOCYTES, EGGS AND EMBRYOS OF *XENOPUS LAEVIS*

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DECLARATION

All the experimental results presented in this thesis were obtained solely by the author.

A. Chambers

LIST OF ABBREVIATIONS:

DNA = Deoxyribonucleic acid

RNA = Ribonucleic acid.

mRNA = messenger RNA.

cDNA = complementary DNA.

bp = base pairs.

kb = Kilobase pairs (1000 bp = 1 kb).

nt = nucleotide.

dNTP = deoxynucleotide triphosphate.

rNTP/NTP = nucleotide triphosphate.

T = deoxythymidine triphosphate / thymidine triphosphate.

A = deoxyadenosine triphosphate / adenosine triphosphate.

C = deoxycytidine triphosphate / cytidine triphosphate.

G = deoxyguanosine triphosphate / guanosine triphosphate.

U = uridine triphosphate.

α ^{32}P rNTP = rNTP with phosphorus - 32 labelled α phosphate.

α ^{32}P dNTP = dNTP with phosphorus - 32 labelled α phosphate.

γ ^{32}P NTP = NTP with phosporus - 32 labelled γ phosphate.

snRNA = small nuclear RNA.

snRNP = small nuclear ribonucleoprotein particle.

U snRNA = member of the class of snRNAs rich in U residues.

SV40 = simian virus 40.

g = gramme.

mg = milligramme.

ug = microgramme.

BSA = bovine serum albumin.

Ci = curie.

EDTA = sodium ethylenediaminetetraacetate.

EGTA = ethyleneglycol - bis - (beta - aminoethyl ether) - N, N, N', N - tetraacetic acid.

rpm = revolutions per minute.

SDS = sodium dodecylsulphate.

TEMED = N, N, N', N' - Tetramethyl-ethylendiamine.

Tris = Tris (hydroxymethyl) aminomethane.

SUMMARY

The general mechanism used to make the 3' ends of eukaryotic mRNAs involves 3' cleavage of a precursor mRNA followed by the addition of a poly - A tail to the newly formed mRNA 3' end. The 3' ends of histone mRNAs are generated by a 3' cleavage reaction but these mRNAs are usually not polyadenylated. An exception to this rule is in the Xenopus oocyte where polyadenylated histone transcripts do exist. These adenylated histone transcripts become de - adenylated as the oocyte matures into an egg. Changes in the rate of histone protein translation also occur during Xenopus development, with increases in the translation rate in the egg over the oocyte and in the embryo over the egg. This thesis describes experiments in which histone precursor mRNAs, made in vitro, were injected into oocytes, eggs and embryos to determine whether 3' cleavage of precursor mRNAs also varied during early development. It was confirmed that histone precursor mRNA was accurately and efficiently 3' cleaved after injection into the oocyte nucleus. Accurate but less efficient 3' cleavage occured on injection of histone precursor mRNAs into eggs and embryos. A polyadenylation activity was discovered in eggs and embryos which added an A tail directly to the 3' ends of injected precursor mRNAs. Experiments to investigate this polyadenylation activity were performed. The significance of the observed histone 3' processing activities is discussed in relation to possible mechanisms for the increase in translation of histone proteins during early development.
Experiments using the oocyte as an assay system, to investigate 3' end formation of polyadenylated mRNAs, were also performed. In DNA injection experiments the oocyte was shown to correctly generate the mouse β globin mRNA 3' end. In injection experiments using artificial precursor mRNAs to both mouse globin and Xenopus heat shock mRNAs no 3' cleavage was detected.

1. INTRODUCTION

INTRODUCTION

1.1 FOREWORD

In the eukaryotic cell mRNA molecules are the link between the genetic information in the nuclear DNA and the mechanisms to translate that information into proteins in the cell cytoplasm. This central role within the cell makes an understanding of mRNA metabolism vitally important to an understanding of cell activity at the molecular level. One of the most interesting aspects of mRNA metabolism is the series of RNA processing reactions necessary to generate a mature mRNA molecule. The most studied of these processing reactions has been RNA splicing, this is the mechanism by which intron sequences are removed from a precursor mRNA and exon sequences are joined to form a mature mRNA. A less studied, but now regarded as increasingly important, area of mRNA metabolism is the way in which the 3' ends of mRNAs are formed. Research into this area has already shown that this seemingly mundane event is actually the result of a highly accurate and complex RNA processing activity.

This thesis describes the results of a study of mRNA 3' end formation based on microinjection of DNA and RNA into oocytes, eggs and embryos of Xenopus laevis. The results are presented in two distinct but closely related sections. In the first results section work on histone mRNA 3' end formation and how this varies at different stages in Xenopus development is described. The second results section describes an investigation into 3' end formation of polyadenylated, non - histone, mRNAs using the Xenopus oocyte as an assay system.

The following introduction consists of a review of the general background information followed by a more specific introduction to the two sections of the project.

1.2 EUKARYOTIC TRANSCRIPTION TERMINATION

The generation of the 5' end of eukaryotic mRNAs is a straightforward process involving the initiation of transcription at a site at the 5' end of the transcription unit (the cap site) which forms the 5' end of the mRNA. The mRNA 5' end generated in this way is very rapidly modified by the addition of a cap structure (for review see Nevins, 1983). This cap structure is a modified nucleotide which enhances the stability of the mRNA and is also necessary for efficient RNA splicing (Konarska et al, 1984). There is no evidence for transcription starting upstream of the cap site to generate a precursor mRNA which bears a 5' extension and which is processed to form the 5' end of the mature mRNA. The possibility that a 5' processing activity may exist was tested in Xenopus oocyte nuclei. In these experiments an artificial histone H4 mRNA with an extension at the 5' end was made *in vitro*. On injection into the oocyte nucleus this did not undergo a 5' processing reaction (Georgiev et al, 1984).

The ways in which the 3' ends of mRNAs are formed are more complicated. It might seem logical to generate the 3' end of the mRNA by terminating transcription at the mRNA 3' end site, this however does not seem to be the strategy used for transcripts from most eukaryotic genes, one exception is transcripts from

certain yeast genes. The yeast iso-1-cytochrome c gene contains a region immediately downstream of the coding region which directs transcription termination (Zaret and Sherman, 1982). From the sequence of this region and from the sequences of other yeast genes the following consensus terminator was derived :

5' 3'

T A A T A G T

T A G - (1-140 nt- T rich) -T A G - T A T G T-(A-T rich)-T T T--

T G A

Termination

Transcription terminates after transcribing the sequences shown. The resulting transcripts are then polyadenylated directly. A yeast mutant, cyc1-512, which lacks this terminator region in the cytochrome c gene, produces longer than normal transcripts with 3' ends in downstream positions. These longer transcripts are polyadenylated suggesting a possible link between transcription termination and polyadenylation rather than polyadenylation being controlled by sequences close to the normal mRNA 3' end position.

The sequence T T T T T A T A has also been suggested as a possible yeast terminator (Henikoff et al, 1983, Henikoff and Cohen, 1984). This sequence, present in certain yeast genes, was seen as a control sequence directing transcription termination to occur at some distance downstream.

A second example of transcription termination directly generating the 3' ends of transcripts has been discovered recently (Rohrmann et al, 1986). An *in vitro* transcription system was prepared from vaccinia virus virions. When vaccinia

virus template DNAs were added to this system, transcripts, the sizes of mature vaccinia transcripts, were produced. The 3' ends of these vaccinia transcripts appeared to be generated directly by transcription termination downstream of a regulatory signal. The regulatory signal contained the sequence T T T T T A T.

For the other non - yeast transcription units so far studied transcription does not terminate at the site of the mRNA 3' end, instead it continues in the downstream direction and terminates some distance away from the 3' end of the final exon. Downstream transcription termination was first observed for viral transcription units including the SV40 late transcription unit (Ford and Hsu, 1978), the adenovirus type 2 major late transcription unit (Nevins and Darnell, 1978) and early regions 2 and 4 of adenovirus type 2 (Nevins, 1980). In these studies although it was shown that transcription continues past the mRNA 3' end position no downstream terminator sequences were identified. An attempt to map the transcription termination site of the mouse β (major) globin gene has been made following the observation that in nuclei from induced mouse erythroleukemia cells transcription proceeds past the mRNA 3' end position of this gene (Hofer and Darnell, 1981). The mapping was a technically difficult procedure because it involved labelling nascent run-on transcripts over short time periods to identify very short-lived precursors. These experiments showed that transcription terminates gradually over a termination region rather than at a precise site. This region is 700 - 2000 nt downstream from the mRNA 3' end position (Salditt - Georgieff et

al, 1983, Citron et al, 1984), it is characterised by three repeated AATAAA sequences at the 5' end and a potential stem and loop structure followed by 15 T residues at the 3' end. This transcription terminator has been shown to cause transcription termination when inserted into the adenovirus E1A gene (Falck - Pedersen et al, 1985). In these experiments it was found that the operation of the terminator depended on it's orientation. Only when it was inserted in the E1A gene in the same orientation, relative to transcription, as it was in the ß globin gene, would it cause termination. Upstream sequences, including the sequences around the ß globin mRNA 3' end site, were also necessary for the terminator to function. This suggested that it may be necessary for the mRNA 3' end position to be selected before transcription termination can occur. If the potential mRNA 3' end site is missing and cannot be selected then transcription does not terminate at the terminator sequence. These results demonstrated that a terminator sequence from one gene can function when inserted into a different gene. The mechanism of termination must be sequence specific rather than being directed by some overall effect of chromatin structure. Other genes where transcription has been shown to terminate downstream of the mRNA 3' end position include the following :

(a) The rat calcitonin gene (Amara et al, 1984). Here one gene has the potential to generate two different mRNAs depending on the tissue. In thyroid C cells calcitonin mRNA is produced whereas in brain, spinal cord and nerve ganglia calcitonin gene related peptide (CGRP) mRNA is produced. The 2 mRNAs result

from a combination of differential mRNA 3' end formation and exon splicing. To form calcitonin mRNA, the mRNA 3' end is produced at the end of exon 4 and this is spliced to exons 1, 2 and 3. To produce CGRP mRNA, the mRNA 3' end is generated at the end of exon 6 and exons 5 and 6 are spliced to exons 1, 2 and 3. In both cases transcription terminates about 1 Kb downstream of exon 6.

(b) The mouse dihydrofolate reductase gene (Frayne et al, 1984). This gene has the potential to generate 7 different mRNAs each with the 3' end in a different position. In each case transcription continues downstream through all 7 mRNA 3' end positions and terminates in a 900 nt region located about 1 Kb downstream from the 7 th 3' end site.

(c) The mouse α amylase gene (Hagenbuchle et al, 1984). Here transcription terminates over a fairly long region between 2.5 and 4 Kb downstream of the mRNA 3' end position.

(d) The chicken ovalbumin gene (LeMeur et al, 1984). Greater than 90 % of transcripts from this gene terminate in a 170 nucleotide region 900 bp downstream from the major mRNA 3' end position. Interestingly this termination region contains two sequences AATTTATA and TATTTATA which show homology to the sequence TTTTTATA which has been proposed to be an essential feature of the yeast transcription terminator.

These data suggest a picture of transcription termination occuring rather gradually over a long terminator region rather than at a precise site. Recent work on the human gastrin gene contradicts this model (Sato et al, 1986). This work identified a specific terminator element located 192 nt downstream from the

mRNA 3' end position. This element consists of 32 nucleotides, 27 T residues, interspersed with 5 A,s. The element is orientation specific with respect to the direction of transcription and it causes termination without a preceeding potential mRNA 3' end site. The A - T rich composition of this element again makes it similar to the yeast transcription terminators.

All the transcription units discussed so far produce mRNAs that are polyadenylated. It would be tempting to think that the strategy of downstream transcription termination followed by an RNA processing step was adopted because of some constraint of the polyadenylation system, for example polyadenylation could be obligatorily coupled to a 3' cleavage reaction. However a similar strategy is almost certainly used to make the 3' ends of histone mRNAs, the classic example of non - polyadenylated mRNAs (Greenberg and Perry, 1972, Adesnik and Darnell, 1972) [see section 1.3].

The overall conclusion from the data presented in this section is that transcription termination downstream from the mRNA 3' end position is probably a general eukaryotic mechanism, although there are certain exceptions as discussed above. Clearly however much remains to be discovered about the mechanism of transcription termination. Transcription termination downstream of the mRNA 3' end position generates a precursor mRNA which undergoes processing at the 3' end to generate the mature mRNA. the systems used to make the 3' ends of mRNAs are discussed in the following sections.

1.3 HISTONE mRNA 3' END FORMATION

The mechanism by which the 3' ends of histone mRNAs are formed is better understood than the mechanism for making the 3' ends of polyadenylated mRNAs. This is largely due to the existence of a good model system based on the injection of histone DNAs and artificial precursor mRNAs into the Xenopus oocyte.

The majority of histone genes are the so-called replication-dependent variants, they are expressed during the S phase of the cell cycle and produce histone proteins which complex with newly replicated DNA (for reviews see Hentschel and Birnstiel, 1981; Old and Woodland, 1984). These genes do not contain introns and they produce mRNAs which are not polyadenylated in somatic cells (Greenberg and Perry, 1972; Adesnik and Darnell, 1972). The latter property immediately sets them apart from the majority of eukaryotic mRNAs which are polyadenylated. In this section I will review the mechanism by which the 3' ends of these replication-dependent, non - polyadenylated histone transcripts are made.

a. The 3' ends of histone mRNAs can be generated by an RNA processing reaction.

The 3' end of an mRNA might be made either directly by transcription termination or as a result of processing of a longer precursor mRNA. Initially it seemed an attractive hypothesis that histone mRNA 3' ends were formed directly by transcription termination. This hypothesis was considered because histone genes contain a highly conserved region of dyad symmetry

at their 3' ends (3' terminal palindrome). It was thought that this region might form a stem and loop structure in the newly transcribed mRNA and function in a similar way to a bacterial transcription terminator. It has now been shown that mechanisms exist both in Xenopus oocytes and in *in vitro* systems, made from cell lines, that can 3' process histone precursor mRNAs. These mechanisms would not be necessary if the mRNA 3' end was made directly by transcription termination. In these experiments artificial RNAs were made to resemble genuine histone precursor mRNAs. Each artificial precursor mRNA was a capped RNA transcribed from a histone gene, with sequences downstream from the mRNA 3' end position included. The precursor mRNA was then added back to either an *in vivo* or *in vitro* situation to see whether 3' processing would occur.

This type of experiment was first reported for the sea urchin H2A histone mRNA (Birchmier et al, 1984). Precursor mRNAs were made by transcription of DNA templates using E.coli RNA polymerase. On injection of transcripts with a 230 nucleotide 3' extension into the oocyte nucleus, RNAs with their 3' end in the correct position were generated. The shorter RNAs were the result of an RNA processing activity removing the sequences downstream of the mRNA 3' end position, from the precursor mRNA.

A report soon afterwards (Krieg and Melton, 1984) described a similar experiment in which a chicken H2B histone precursor mRNA was made using SP6 polymerase to transcribe a linear DNA template. On injection of this precursor mRNA into the

oocyte nucleus RNAs 3' processed to the correct site were detected.

Later work (Georgiev et al, 1984) demonstrated that a Xenopus H4 precursor mRNA, made using SP6 polymerase, was 3' processed when injected into the oocyte nucleus. This work also showed that a 5' cap on the precursor mRNA was necessary for efficient 3' processing.

These experiments demonstrated that 3' processing of histone transcripts can occur in the oocyte nucleus and therefore might be involved in the generation of endogenous oocyte histone mRNAs. Further work using *in vitro* processing systems has shown that the mechanism is more widespread and has revealed information as to how 3' processing is achieved.

The first *in vitro* processing system for histone mRNAs was developed using a nuclear extract from Drosophila cells (Price and Parker, 1984). Transcription of a Drosophila H3 histone gene in this extract led to the formation of precursor mRNAs which were 3' processed by a magnesium- dependent processing activity.

An *in vitro* system that 3' processes mouse H4 histone precursor mRNAs has also been developed (Gick et al, 1986). This system was made from a HeLa cell nuclear extract. The added H4 precursor mRNAs were processed by a magnesium-dependent processing activity. In this system , in the presence of EDTA, it was possible to detect both the processed H4 RNA and RNAs representing the cut - off 3' extension. This result indicated that processing was achieved by endonucleolytic cleavage rather than by a processive exonuclease. The absence of magnesium ions

was essential to stabilise the cut - off portion of the RNA against degradation. In the oocyte microinjection experiments, described above, it was not possible to detect the discarded RNA sequences, this was probably because they were uncapped and quickly degraded. The cut - off 3' extension detected in the *in vitro* experiments did not appear as a single fragment the size of the extension, instead it was represented by several smaller fragments. This result suggested that 3' processing might involve several simultaneous endonucleolytic cleavages. Further work with this system should allow the molecular details of the processing mechanism to be elucidated.

b. Sequences controlling 3' processing of histone mRNA.

Histone genes contain 2 conserved sequence elements around the mRNA 3' end position. One of these elements is a highly conserved region of dyad symmetry immediately upstream of the mRNA 3' end position (3' terminal palindrome). When transcribed into mRNA this element could form a stem and loop structure. The sequence of the element is very highly conserved in replication-dependent histone genes from a wide range of organisms, these include the sea urchin, Drosophila, Xenopus, chicken, mouse and man (see Birchmier et al, 1983). Histone sequences from these organisms were compared to derive the following consensus for this element :

```
5'          T              A          3'
  A A C G G C C C T T T T C A G G G C C A C C A
      ---------->      <-------------    ↑
                                        mRNA 3' end
```

The mRNA 3' terminus is produced as indicated 4 nucleotides downstream from the end of this element.

The other conserved sequence element is found 10 - 17 nucleotides downstream from the terminal palindrome. It consists of a purine rich sequence which is less well conserved than the terminal palindrome. In sea urchin histone genes this takes the form :

5' 3'

C A A G A A A G A

(Busslinger et al, 1979). In vertebrate histone genes the consensus :

5' 3'

A A A A G A G C T G

has been derived.

The two sequences share the A A A G A core sequence (Birnstiel et al, 1985).

The two conserved sequence elements, located on either side of the mRNA 3' end position, are vitally important in the 3' processing mechanism. The 3' processing of sea urchin histone gene transcripts from the h22 cluster (Clarkson et al, 1976) in the Xenopus oocyte has been the major model system. Early experiments in which h22 DNA was injected into the nucleus of the oocyte showed that the H2A and H2B genes produced transcripts with correctly positioned 3' termini. It was also shown that the sea urchin H3 gene produced longer than usual, read through, transcripts (Probst et al, 1979. Hentschel et al, 1980). These facts have been exploited, using the H2A gene to identify the

sequences necessary for processing, and the H3 gene to identify a processing factor from sea urchin embryos.

Experiments in which a series of deletion and insertion mutants of the H2A gene were analysed for 3' processing in the oocyte nucleus showed the importance of the terminal palindrome (Birchmier et al, 1982). In deletions where the palindrome was removed correct H2A mRNA 3' ends were not formed, instead read through transcripts resulted. The palindrome alone was not sufficient to direct 3' processing, downstream spacer sequences were also required, these included the downstream conserved element.

Further work showed that the terminal palindrome plus the downstream C A A G A A A G A element were together sufficient to direct accurate 3' end formation but 80 nt of spacer sequence from adjacent to the gene was required before this occured with optimum efficiency (Birchmier et al, 1983). In these experiments point mutations were made in the terminal palindrome which altered the sequence of the element and would also have prevented it from forming a stem and loop structure. These mutations abolished 3' processing, this might have been because they changed the sequence of the mRNA 3' end which was itself necessary for processing or because they prevented the formation of the RNA secondary structure. In the latter case the secondary structure rather than the exact sequence would be of prime importance. Mutants with 2 complementary changes in the terminal palindrome, which although having a different 3' sequence retained the ability to form a stem and loop structure, were

tested for 3' processing. These mutants were processed at the wild type level which suggested that the secondary structure formed at the mRNA 3' end was of prime importance. Subsequent work on the mechanism of histone mRNA 3' processing has revealed that this conclusion is misleading.

Experiments in which the sea urchin H2A gene was used to make synthetic precursor mRNAs confirmed that the conserved elements were required for an RNA processing reaction (Birchmier et al, 1984). An H2A precursor mRNA with 2 base changes in the terminal palindrome was not processed when injected into the oocyte nucleus. Similarly a precursor in which the terminal palindrome was intact but the downstream element was removed was not processed. Only when both elements were intact did 3' processing occur, furthermore 200 nt of spacer sequence, from adjacent to the gene, was also necessary for processing to reach an optimum level.

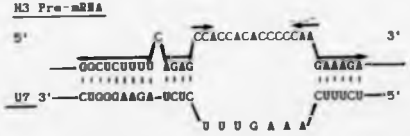
c. The histone mRNA 3' processing machinery.

The role that the two conserved sequence elements play in 3' processing became clear as details of the 3' processing machinery were discovered. The details were obtained mainly as a result of using the sea urchin H3 gene, from the h22 cluster, in oocyte injection experiments. On injection of h22 DNA it was observed that the H3 gene produced read through transcripts (Hentschel et al, 1980). This observation was the basis for a series of experiments that succeeded in identifying a processing factor. Initially it was found that a chromosomal salt wash fraction from sea urchin embryos, microinjected into the oocyte cytoplasm,

prior to the nuclear injection of H3 DNA, would correct the deficiency in processing of the H3 transcripts (Stunnenberg and Birnstiel, 1982). The factor was found to sediment at 12S on sucrose gradient centrifugation. The active component was a small, non - polyadenylated RNA about 60 nt long (Galli et al, 1983). The RNA was identified as a snRNA which was probably active as part of a snRNP. Injection of the snRNA alone into the oocyte cytoplasm, one day prior to H3 DNA injection, was sufficient to rescue processing of the H3 transcripts. It was suggested that the snRNA associated with Xenopus snRNP proteins in the oocyte cytoplasm and then migrated into the nucleus, its site of action. The snRNA was cloned as cDNA and a number of cDNA clones were sequenced (Strub et al, 1984). The snRNA was termed U7 because in the form of a snRNP it was precipitable by anti - Sm antibodies, this is a characteristic of the U series of snRNAs. The sequence showed that it was rich in U residues, another characteristic of the U snRNAs. Of the cDNA clones sequenced one was 57 nt long whilst the others were slightly shorter. However, as a consequence of the cDNA cloning protocol, the exact size of the U7 snRNA could not be determined. The sequence of the U7 RNA was very revealing. The 5' end of the U7 snRNA was found to contain extensive sequence homology to the two conserved sequence elements that flank the sea urchin histone mRNA 3' end position. 13 out of 16 nt from the terminal palindrome and 6 out of 9 nt of the CAAGAAAGA sequence were found to be complementary to sequences at the 5' end of the U7 snRNA. This observation led to the proposal that the 5' end of the U7

snRNA could base pair with the conserved sequences flanking the histone mRNA 3' end position as an essential step in the processing reaction.

Proposed base-pairing between the sea urchin H3 precursor mRNA and the U7 snRNA:



The evidence that both elements are necessary for processing of H2A mRNA supports this proposal (Birchmier et al, 1984). These experiments also suggested that further spacer sequence is also required for efficient processing. These additional spacer sequences could not exert their effect by base pairing to the U7 snRNA. The remainder of the U7 snRNA, not involved in base pairing, would be responsible for binding the snRNP proteins.

Evidence for this model of a U7/Histone precursor mRNA interaction leading to 3' processing comes from the following work (Georgiev and Birnstiel, 1985). Various linker scan, deletion and insertion mutants of the H3 gene were prepared, these were then injected into oocytes previously injected with the U7 snRNA. Unaltered H3 genes produced transcripts with correct 3' termini. Genes with alterations to either of the two conserved sequence elements produced read through transcripts.

Mutations which added extra sequence between the two elements were equally deleterious abolishing all 3' cleavage. This result suggested that not only were both intact elements necessary for 3' cleavage they also had to be in the correct position in relation to each other. Similar experiments were carried out using artificial H3 precursor mRNAs synthesised using SP6 polymerase. On injection of a H3 precursor with a 240 nt 3' extension into oocytes preinjected with U7 RNA, correctly 3' cleaved H3 transcripts were detected. Mutant RNAs with changes similar to those of the mutant DNAs were then synthesised and tested for 3' processing. Mutations that affected the 3' terminal palindrome, the CAAGAAAGA sequence or the distance between the 2 elements abolished 3' cleavage, results similar to those from the mutant DNA injections. Some differences in the importance of spacer sequences downstream from the CAAGAAAGA sequence were observed between the DNA and RNA injection experiments. Removal of spacer sequences to a point just downstream of the CAAGAAAGA sequence abolished processing of precursor mRNAs but was only a strong down mutation in the DNA injection experiments.

This work confirmed that for U7 mediated cleavage of H3 mRNAs the same sequences were required as previously identified for H2A 3' cleavage. This suggests that the cleavage mechanism for histone mRNAs other than the H3 could well be mediated via a similar interaction between the precursor mRNA and a U7 equivalent.

Final proof that the U7 snRNA component of the active sucrose gradient / RNA gel fraction was the vital factor in H3 3' cleavage, in oocyte injection experiments, has recently been obtained (Strub and Birnstiel, 1986). Before this result the formal possibility that 3' cleavage was mediated by some very minor, unidentified component of the same fraction remained. A U7 cDNA probe was used to isolate a gene cluster containing 5 U7 genes from the sea urchin Psammechinus miliaris (Dalorenzi et al, 1986). One of these genes was linked to a Xenopus U2 gene promoter and in microinjection experiments in Xenopus oocytes was shown to yield authentic U7 RNA transcripts. The Xenopus U2 promoter was necessary in order to obtain expression of the sea urchin U7 gene in the oocyte. Experiments were then carried out in which the U2/U7 hybrid gene was coinjected with the sea urchin H3 gene from the h22 cluster. In these coinjection experiments the U7 RNA transcribed from the hybrid U2/U7 gene was sufficient to bring about the 3' cleavage of the transcripts from the H3 gene. This result confirmed that the U7 snRNA, probably in the form of an snRNP, was indeed the processing factor in the previously identified active fraction.

The use of the hybrid U2/U7 gene was the basis for recent work that confirmed that the interaction between the U7 snRNA and the H3 precursor mRNA is by base - pairing (Schaufele et al, 1986). Mutations were made in the CAAGAAAGA sequence of the sea urchin H3 gene. When these mutants were co - injected with the U2/U7 hybrid, into Xenopus oocytes, correct H3 3' termini were not formed, the potential base - pairing interaction between the

U7 snRNA and the H3 precursor mRNA was disrupted. When complementary mutations were made in the U7 gene, that allowed the U7 snRNA to base - pair with the mutant H3 precursor mRNA, H3 mRNA 3' end formation was restored.

Evidence that U7, or U7 equivalent, -mediated 3' cleavage of histone precursor mRNAs is a general mechanism has been obtained from work using a HeLa cell nuclear extract as a RNA 3' processing system (Gick et al, 1986). This system cleaves added mouse H4 precursor mRNAs at the mRNA 3' end position. An anti - Sm antibody, which precipitates a number of U snRNPs including the U7 snRNP, was found to inhibit H4 3' cleavage in this system. When an extract of snRNPs was added back to the system the 3' cleavage mechanism was restored. This strongly suggests that the 3' cleavage mechanism used in HeLa cells is again dependent upon U snRNPs.

By combining the data reviewed in this section a current model for histone mRNA 3' end formation can be formulated. Transcription from the histone promoter proceeds in the 3' direction through the coding region of the gene and past the position where the mRNA 3' end is formed. Transcription terminates at heterogeneous sites downstream from the mRNA 3' end position in the spacer DNA, this generates capped precursor mRNAs with extensions at the 3' end. Each precursor mRNA undergoes rapid 3' processing to generate the mature mRNA. The two conserved sequence elements that flank the mRNA 3' end position direct the processing machinery where to form the mRNA 3' end,

this involves base pairing of these elements with a U7 (or U7 equivalent) snRNA, which is part of a snRNP particle. The presentation in a stem and loop structure of some of the sequences to base pair with U7 may be essential for this interaction. The precursor mRNA undergoes 3' processing which involves one or more endonucleolytic cleavages. The 3' extension removed in the processing reaction is uncapped and is quickly degraded. The 3' processed mRNA has a cap at the 5' end and a non - polyadenylated 3' end. The 3' terminal palindrome may then reform a stem and loop structure to stabilise the 3' end of the molecule against exonuclease degradation. The processed mRNA is then transported out of the nucleus.

Although the above is a good basic model for histone mRNA 3' processing a number of questions remain to be answered, some of these are discussed below :

1. The model implies that the sequence of the 3' terminal palindrome is of prime importance in 3' processing although its ability to form a stem and loop structure may also be important. The experiments described (Birchmier et al, 1983) suggested that the secondary structure of the terminal palindrome was more important than its exact sequence. Here complementary mutations that changed the sea urchin H2A 3' terminal palindrome sequence but still allowed a stem and loop structure to form did not abolish 3' cleavage. However these results are misleading because the changes made did not affect the basic pattern of base pairing between the H2A precursor mRNA and U7.

2. Both DNA (Birchmier et al, 1983) and RNA (Birchmier et al, 1984) injection experiments, using the sea urchin H2A gene, suggested a role for spacer sequences downstream of the CAAGAAAGA element in 3' processing. The is no role for these spacer sequences in the current model.

3. In oocyte microinjection experiments using the h22 sea urchin cluster the H2A and H2B transcripts showed normal 3' processing whereas the H3 transcripts showed aberrant 3' processing. All 3 genes possess the 2 conserved sequence elements flanking the mRNA 3' end position. Something else, presumably the downstream spacer sequences , must determine that the H2A and H2B transcripts are recognised by the oocyte processing machinery whereas the H3 transcripts are not.

4. It has been shown that a 5' cap structure is necessary for efficient histone mRNA 3' processing but that the exact sequence at the mRNA 5' end is not important (Georgiev et al, 1984). How does this fit into the model ?

5. Although the U7 snRNA has been shown to be essential for 3' processing details of how this is achieved are not known. Does the U7 snRNA arrange the precursor mRNA in a particular secondary structure so that a 3' processing reaction is energetically favoured ? Does some component of the U7 snRNP particle catalyse the 3' processing reaction ? Or does U7 act as a connection between the precursor mRNA and a separate protein component ?

d. Polyadenylated histone mRNAs.

Although the majority of histone transcripts are non - adenylated examples of histone mRNAs in the polyadenylated class of mRNAs are known. Some of these examples are discussed below :

In the oocytes of amphibians histone mRNAs are known to be polyadenylated. In the newt oocyte approximately 50% of the histone transcripts are adenylated (Ruderman and Pardue, 1978). This adenylated histone mRNA includes transcripts coding for all 5 of the histone proteins.

H4 and H3 mRNAs from the Xenopus oocyte have been cloned into cDNA using a protocol based on an oligo - dT primed reverse transcription (Turner and Woodland, 1982). The cloning of these mRNAs in this way indicated that they were polyadenylated. Further work investigated the length of the A tracts on H4 mRNAs in Xenopus oocytes and eggs (Ballantine and Woodland, 1985). This work showed that in oocytes the H4 mRNAs possessed poly - A tracts of various lengths from 0 - 14 A residues at their 3' ends. The most common length was 12 A residues and few of the molecules seemed to possess tracts longer than 14 A's. This work also showed that on maturation of the oocyte into an egg the short A tracts were completely removed. The H4 and H3 cDNA clones from the oocyte had the characteristics of typical non - adenylated histone transcripts, they possessed a 3' terminal palindrome and they did not possess a AATAAA hexamer near to the 3' end. The poly - A tail appeared to be added on to the

transcripts at the normally non - polyadenylated mRNA 3' end position. Primer extension analysis of the adenylated H4 mRNAs indicated that they were transcripts from the same genes that also produced typical non - polyadenylated transcripts. There was no evidence for a subset of polyadenylated mRNA producing genes transcribed only in the oocyte. From this evidence it appears that in the oocyte histone precursor mRNAs are transcribed from the histone genes that later in development produce non - adenylated transcripts. These precursor mRNAs undergo 3' cleavage in the usual way to generate a non - polyadenylated 3' end. The poly - A tract is then added post - transcriptionally.

Other histone mRNAs are produced from genes that always produce polyadenylated transcripts. In yeast and Tetrahymena the histone mRNAs are polyadenylated (Fahrner et al, 1980, Bannon et al, 1983). The yeast H4 gene does not contain the typical 3' terminal palindrome and it has the sequence AATATT, similar to the polyadenylation hexamer, located near to the mRNA 3' end.

The chicken and duck H5 genes also produce polyadenylated mRNAs (Krieg et al, 1982, Doenecke and Tonjes, 1984). These genes lack the typical histone 3' terminal palindrome. Neither has a perfect match to the polyadenylation hexamer, although the chicken H5 gene has the related TATAAA sequence. The chicken H5 gene has been injected into the Xenopus oocyte nucleus. Here it produces transcripts with the 3' end in the correct position however this end is not polyadenylated (Wigley et al, 1985).

The chicken H3.3 and H2AF histone genes also produce polyadenylated mRNAs whilst lacking both the histone 3' terminal

palindrome and a version of the polyadenylation hexamer (Engel et al, 1982, Harvey et al, 1983). It has been suggested that the H3.3 gene may not actually produce transcripts polyadenylated at the 3' end, an A tract within the mRNA may have been responsible for its binding to oligo - dT cellulose (Engel et al, 1982).

1.4 THE FORMATION OF POLYADENYLATED mRNA 3' ENDS

The majority of mRNAs in the eukaryotic cell have polyadenylated 3' ends. The details of the mechanism by which these 3' ends are formed remain unresolved. From the evidence so far accumulated it appears that polyadenylated mRNAs share a common basic 3' processing mechanism but the relative importance of different control sequences may vary dependent on the gene and the cell type. I will start by reviewing the sequences involved in controlling 3' end formation of polyadenylated mRNAs and then go on to discuss the processing machinery which interacts with these sequences.

a.) Control sequences

The most obvious conserved feature around polyadenylated mRNA 3' end positions is the hexamer AATAAA in the DNA sequence transcribed to AAUAAA in the precursor mRNA. This hexamer was first identified on the basis of sequence comparisons, initially between the rabbit β globin and the mouse immunoglobulin light chain mRNAs (Proudfoot and Brownlee, 1974). A slightly later sequence comparison showed that this sequence was located about 20 nt upstream of the 3' end position of 6 different polyadenylated mRNAs (Proudfoot and Brownlee, 1976). Because of the conserved nature of this hexanucleotide and because of its location it was suggested to be involved in forming the 3' end of the mRNA. As the sequences of more genes have become available the presence of this hexamer, 12 - 30 nt upstream from the mRNA

3' end position, has been remarkably consistent. Genes which lack the exact hexamer usually have a closely related sequence in the same position. A survey of available sequences (Birnstiel et al, 1985) showed that as well as the AAUAAA hexamer the following sequences could be found upstream from mRNA 3' end positions and about the same distance away as the usual hexamer :

AAUUAA, AAUACA, AAUAAU, AAUAAC, CAUAAA, AUUAAA, AGUAAA, UAUAAG, AAUAUA

It remains unclear why certain changes in this hexamer are tolerated and others are not.

The AAUAAA hexamer has been shown to be functionally important in mRNA 3' end formation (Fitzgerald and Shenk, 1981). In these experiments the affects of deletions around the SV40 late transcription unit 3' end position were investigated. The experimental system used was CV - 1P cells infected with virus. In the SV40 late transcription unit the AAUAAA hexamer is located 12 nt upstream from the mRNA 3' end. When the hexamer was deleted the formation of adenylated 3' ends at the normal site was abolished. When sequences between the hexamer and the mRNA 3' end position were deleted polyadenylated mRNAs were produced with their 3' ends at a different site to normal but within a fixed distance of the hexamer. These results suggested that the hexamer was important in directing the formation of a polyadenylated mRNA 3' end a fixed distance downstream.

In the human β globin gene the AAUAAA hexamer is normally located upstream from the mRNA 3' end position. It has been shown that a U to C conversion in the hexamer causes β thalassemia. Patients with this thalassemia posess a unusually large β globin mRNA. This large RNA is globin precursor mRNA that accumulates because the U to C conversion in the hexamer decreases the efficiency of β globin mRNA 3' end formation (Orkin et al, 1985), this is good evidence of the importance of the hexamer in producing mRNA 3' ends *in vivo*.

The importance of the hexamer has also been demonstrated *in vitro* (Zarkower et al,1986). In these experiments a HeLa cell nuclear extract was used as a processing system for artificial SV40 late precursor mRNAs. These RNAs were cleaved and polyadenylated in the extract. Mutant precursor mRNAs in which bases in the AAUAAA hexamer were changed were not 3' cleaved or polyadenylated in the extract.

These experiments have demonstrated the importance of the AAUAAA hexamer in mRNA 3' end formation, however as more gene sequences became available it was quickly realised that this could not be the only element directing mRNA 3' end formation. The AAUAAA sequence is found in positions nowhere near mRNA 3' end sites, for example it is found in the middle of the early region of the SV40 genome (Fiers et al, 1978) and in the adenovirus type 12 E1A transcription unit (Perricaudet et al, 1980).

The second conserved sequence element to be recognised was the CACUG sequence adjacent to the mRNA 3' end position in

several eukaryotic genes (Benoist et al, 1980). A more detailed sequence comparison of 61 vertebrate genes further defined this sequence (Berget et al, 1984). Here the element was defined as CAYUG, where Y represents any nucleotide. This element was found to be located either immediately upstream or immediately downstream of the mRNA 3' end position in many genes. Genes can be divided into two classes dependant upon the position of the CAYUG element relative to the mRNA 3' end. Genes with the element upstream of the mRNA 3' end were put into class 1, exemplified by the SV40 early transcription unit. Genes with the element downstream of the mRNA 3' end position were put into class 2, exemplified by the SV40 late transcription unit. A model was suggested in which the AAUAAA hexamer and the CAYUG element would together direct mRNA 3' end formation, this could possibly be achieved by base pairing between these sequences in the precursor mRNA and the U4 snRNA.

A further weakly conserved sequence element has also been identified downstream from mRNA 3' end positions and has been implicated in mRNA 3' end formation. The element, termed the G/T cluster, was identified on the basis of its functional importance rather than from sequence comparisons, it forms a region downstream from the mRNA 3' end position rich in G and T residues.

A large body of evidence exists showing the importance of sequence elements downstream of the mRNA 3' end position in 3' end formation. 3 types of systems have been used in these studies: cultured cells, injection into the Xenopus oocyte and *in vitro*

systems. I will review the evidence from each of these systems in turn.

3' end formation of the Hepatitis B surface antigen mRNA in transfected Cos cells was shown to require 30 nt of sequence from downstream of the AAUAAA hexamer (Simonsen and Levinson, 1983).

Human 293 cells were transfected with a plasmid containing the adenovirus E2A transcription unit, plasmids containing various deletion mutants of this gene were also used for transfection. The wild type gene produced transcripts with the 3' end polyadenylated and at the correct site. Mutants with sequences deleted to within 35 nt downstream of the mRNA 3' end position still produced correct mRNA 3' termini. Mutants deleted to within 20 nt downstream of the mRNA 3' end position did not produce the correct mRNA 3' termini (McDevvit et al, 1984). These results suggested that in addition to the AAUAAA hexamer a second sequence element 20 - 35 nt downstream from the mRNA 3' end position was essential for mRNA 3' end formation.

Further work using the adenovirus E2A transcription unit in human 293 cells identified the downstream sequence element more precisely (Hart et al, 1985). This work narrowed down the element to a region 30 - 35 nt downstream of the mRNA 3' end position. This region contained a match to the sequence T A/G T T T T T which is duplicated several times downstream of this transcription unit. It was also shown that an element located 5 - 18 nt past the mRNA 3' end position in the SV40 early transcription unit was necessary for correct mRNA 3' end

formation. This sequence although G / T rich was not homologous to the essential element in the E2A transcription unit. Interestingly however an SV40 mutant with a deletion in the 5 - 18 nt downstream element, deficient in mRNA 3' end formation, could have the ability restored by the addition of the E2A downstream element. This result suggested that both downstream elements, although not homologous, performed a similar function.

Downstream sequences between 13 and 84 nt past the mRNA 3' end position are essential for the production of correct 3' ends of bovine growth hormone transcripts in transfected Cos cells (Woychik et al, 1984).

Experiments using the rabbit β globin gene in HeLa cells also demonstrated the importance of downstream sequences. Here the AAUAAA hexamer plus 15 nt of downstream sequence was not sufficient for mRNA 3' end formation whereas the hexamer plus 51 nt of downstream sequence was sufficient (Gil and Proudfoot, 1984). The 35 nt region defined in these experiments was rich in G and T residues, it also contained part of the CAYUG sequence.

The effects of downstream deletions on 3' end formation of transcripts from herpes simplex virus type 1 (HSV - 1) and herpes simplex virus type 2 (HSV - 2) genes in HeLa cells has been studied (Mclauchlan et al, 1985). These genes contain a match to the G/T rich sequence Y G T G T T Y Y located about 30 nt downstream from the AATAAA hexamer. When this sequence was deleted the level of correct mRNA 3' ends fell to 1 - 4 % of the wild type level, instead longer, unprocessed, read through transcripts were made.

The 3' end formation of transcripts from the SV40 late transcription unit in COS cells has also been investigated (Sadofsky et al, 1985). Here an essential element with the sequence A G G U U U U U U was found to be necessary for accurate and efficient 3' end formation. This element, which was located 59 nt downstream from the AAUAAA hexamer, shows some sequences complementarity to the U4 snRNA, which suggested that it may be involved in base pairing with U4.

When the 3' end formation of transcripts from the same SV40 late transcription unit was investigated in oocyte microinjection experiments a different result was obtained (Conway and Wickens, 1985). It was shown that a 220 nt DNA fragment spanning the SV40 late mRNA 3' end position, with 79 nt of sequence past this position, was sufficient to direct correct 3' end formation and polyadenylation of transcripts made in the oocyte. By testing deletion mutants a sequence element, located 9 - 26 nt downstream of the AAUAAA hexamer, was shown to be essential for efficient 3' end formation. Without this region, accurate but very inefficient 3' cleavage of the precursor mRNAs occured. This element is in a different position to the one identified in Cos cells.

The overall picture from these results is not clear, probably because of the range of different genes and cell types studied. A number of generalisations can however be made. There does appear to be a requirement for sequences downstream of the mRNA 3' end position, as well as the AAUAAA hexamer, for efficient mRNA 3' end formation. The downstream sequences are rich in G and T residues but are not rigidly conserved, they are

usually located within 50 nt of the mRNA 3' end position. Each gene may possess a number of equivalent downstream elements with the exact role of each depending on the tissue in which the gene is expressed. A recent paper (Mason et al, 1986) presented data that contradicts this model. In the experiments described the Xenopus oocyte microinjection technique was used to analyse the effects of various mutations on 3' end formation of Xenopus β globin transcripts. It was shown that small deletions and linker replacement of sequences downstream of the mRNA 3' end position did not greatly affect the efficiency of mRNA 3' end formation. The sequences that could be changed without reducing the efficiency of mRNA 3' end formation included the CATUG sequence element immediately downstream from the mRNA 3' end position and the downstream G/T rich sequence.

In order to resolve the sequence requirements and the nature of the 3' processing machinery it will probably be best to study the process *in vitro* under controlled conditions. Considerable progress has been made in obtaining mRNA 3' processing systems.

An early attempt to develop an *in vitro* system to study mRNA 3' end formation used a HeLa cell extract (Manley, 1983). SV40 DNAs were used to make run-off transcripts in one batch of the extract, after purification these run off transcripts were added back to a fresh batch of extract. Under the conditions used the RNAs with 3' extensions did not undergo a 3' cleavage reaction, instead poly - A tracts were added directly to the ends of the RNA molecules. This polyadenylation reaction was more efficient when the RNA 3' end was close to the natural mRNA 3' end

position. These results indicated that polyadenylation can be uncoupled from 3' cleavage and the efficiency of polyadenylation may depend on sequences close to the mRNA 3' end position.

Later work demonstrated that a HeLa cell extract was able to cleave and polyadenylate adenovirus transcripts at the L3 site when the transcripts were made in the extract (Moore and Sharp, 1984). This work confirmed that the 3' ends of polyadenylated mRNAs can be made as the result of a 3' cleavage reaction. Purified precursor mRNA added to the extract was not 3' cleaved which led to the suggestion that a secondary strucure in the newly transcribed precursor mRNA was necessary for 3' cleavage. Purified precursor mRNA could have lost the structure as a result of being purified.

The idea that a transcription dependent secondary structure is necessary for mRNA 3' end formation has been ruled out by more recent work (Moore and Sharp, 1985). Here a nuclear extract from HeLa cells was shown to accurately cleave and polyadenylate adenovirus precursor mRNAs at the L3 site. These precursors were made by transcribing DNA templates using SP6 polymerase. About 75% of the added precursor was cleaved and adenylated in 3 hours. No polyadenylation of the precursor RNA without 3' cleavage or 3' cleavage without polyadenylation were detected, suggesting that in this system the 2 activities were linked. The 2 activities were shown not to be obligatorily coupled because in the presence of cordycepin, which blocks polyadenylation, 3' cleavage still occurred. Precursor mRNAs with only 10 nt of downstream sequence past the mRNA 3' end position were not efficiently cleaved

suggesting that, as had been shown in other systems, downstream sequences were necessary for efficient mRNA 3' end formation.

A similar HeLa nuclear extract was used to investigate 3' end formation of SV40 early transcripts. This transcription unit had been shown to require the presence of a downstream sequence element, 5 - 15 nt past the 3' end position, for efficient mRNA 3' end formation *in vivo* (Hart et al, 1985).The HeLa nuclear extract accurately cleaved and polyadenylated added SV40 precursor mRNAs and exhibited the requirement for the downstream sequence element that had been demonstrated *in vivo* (Hart et al, 1985 b.).

The HeLa nuclear extract system will also 3' cleave and polyadenylate artificial SV40 late precursor mRNAs, again exhibiting the same sequence requirements previously determined *in vivo* (Zarkower et al, 1986).

The *in vitro* processing systems now developed should yield much more information on the sequences controlling mRNA 3' cleavage, the link between 3' cleavage and polyadenylation and the sequences which control the polyadenylation reaction.

b. The mRNA 3' processing machinery

The data presented so far has shown that for many eukaryotic genes transcription terminates downstream of the mRNA 3' end position, the precursor mRNA generated then undergoes 3' processing to remove the unwanted downstream sequence and to add

a poly - A tail. 3' processing is controlled by sequences upstream of the mRNA 3' end position such as the AAUAAA hexamer and by sequences downstream of the mRNA 3' end position such as the G/T rich sequences. Some information is available as to the nature of the cellular components that interact with these sequences, but little is known as to how this interaction leads to 3' processing. Small nuclear RNAs (snRNAs) in the form of small nuclear ribonucleoprotein particles (snRNPs) have been implicated in the 3' processing mechanism. snRNAs are small RNA molecules ranging in size from 60 - 220 nt, found in the eukaryotic cell nucleus. snRNAs associate with protein components in particles known as snRNPs, which are regarded as the active form of the snRNAs (for a more detailed review of snRNAs and snRNPs see section 1.5). SnRNPs have been shown to be involved in a number of RNA processing reactions. RNA splicing, the mechanism by which introns are removed from precursor mRNAs and the exons are joined, is dependent upon snRNPs. In this process the snRNPs : U1, U2, U5 and U4/U6 have all been implicated (Rogers and Wall, 1980 : Lerner et al, 1980 : Mount et al, 1983 : Kramer et al, 1984 : Fradin et al, 1984 : Black et al, 1985 : Chabot et al, 1985 : Black et al, 1986).

The mechanism by which the 3' ends of histone transcripts are made involves the U7 snRNA, also probably in the form of a snRNP (Galli et al, 1983 : Strub et al, 1984). In this mechanism the snRNA is thought to interact with the precursor mRNA by base pairing to conserved sequences in the precursor mRNA, close to the processing site.

The suggestion that a snRNA might be involved in 3' processing of polyadenylated mRNAs was initially based on a comparison between conserved sequences around mRNA 3' end positions and the sequences of the U snRNAs (Berget, 1984). Of the U1, U2, U4, U5 and U6 sequences examined it was found that only U4 contained pentanucleotide sequences complementary to both the conserved AAUAAA and CAYUG sequences in the precursor mRNA. These complementary sequences were located in the 5' portion of the U4 snRNA. The 5' region is the region of the snRNA which is not involved in binding snRNP proteins so would be free to base pair with the precursor mRNA, it is also the region which in the U1 RNA is important in base pairing with the 5' splice junction (Kramer et al, 1984). It has been suggested that the downstream G/T rich regions associated with a number of genes could also base pair with the U4 snRNA (Sadofsky et al, 1985). In the SV40 late transcription unit an essential downstream element takes the form AGGUUUUUU which could potentially base pair with a free loop in the U4 snRNA secondary structure.

The evidence to support these suggestions has been mainly obtained through the use of autoimmune sera or monoclonal antibodies against U snRNPs to investigate mRNA 3' processing *in vitro*.

An *in vitro* processing system, made from HeLa cells, was shown to 3' process adenovirus transcripts made in the extract (Moore and Sharp, 1984). Anti - Sm antiserum, which precipitates U1, U2, U4, U5, U6 and U7 snRNPs inhibited 3' processing in this extract as did anti - U1 and anti - La antisera (anti - La serum

precipitates La snRNPs. La snRNAs are transcribed by RNA polymerase III, they are found in the cell nucleus in the form of snRNPs and their role within the cell is not yet clear).

An extract from HeLa nuclei was shown to 3' process artificial adenovirus precursor mRNAs when these were made *in vitro* and added to the extract (Moore and Sharp, 1985). Here too an anti - serum against the U1 snRNP inhibited 3' processing, an anti - U2 antiserum had no affect on the processing reaction.

It is difficult to reach any definite conclusions based on these results because any of the antibodies might also precipitate other, previously unidentified, low abundance snRNPs with common determinants. The anti - Sm activity precipitated a range of U snRNPs so no conclusion as to which, if any, were necessary for 3' processing can be drawn. The essential U snRNP might have been U4 as predicted as this would have been precipitated by the anti - Sm serum. The inhibition by the anti - U1 antibody was suprising because the U1 snRNP was not considered a good candidate for the 3' processing snRNP.

A more recent study has provided better evidence for the involvement of snRNPs in 3' processing (Hashimoto and Steitz, 1986). In these experiments a HeLa nuclear extract was shown to 3' process artificial precursor mRNAs for adenovirus L3 and E2A mRNAs and for SV40 early and late mRNAs. When an anti - Sm antibody was added to the system followed by a ribonuclease T1 digestion specific RNA fragments were protected, these were isolated using protein - A - sepharose and analysed. The results suggested that a protein component, recognised by the anti - Sm

antibody, bound to the precursor mRNA in a specific way. The protected fragments for each precursor mRNA included a region of the precursor containing the AAUAAA sequence. A second antibody directed against the trimethylguanosine cap found at the 5' end of snRNAs gave the same result. These results suggested that a Sm precipitable U snRNP bound to the AAUAAA sequence during RNA 3' processing. Antibodies against U1 and U2 snRNPs did not give this pattern of protection. In the same series of experiments it was shown that severely depleting the levels of U1, U2, U4 or U6 snRNAs in the extract did not inhibit 3' processing. Overall these results suggested that a U snRNP was important for 3' processing a reaction which involved binding of the snRNP to the AAUAAA hexamer in the precursor mRNA. The U snRNP involved was not U1 or U2 and was probably not U4 or U6 either.

A study in which SV40 early and adenovirus L3 precursor mRNAs were used in a HeLa nuclear *in vitro* processing system also suggested that neither U1, U2 or U4 snRNPs were necessary for 3' processing. In these experiments it was first established that the system would 3' process the added precursor mRNAs. Various extracts were then made in which different snRNAs were specifically depleted using oligonucleotide directed cleavage. This procedure involved adding a synthetic oligonucleotide complementary to a specific region of the snRNA to the extract in the presence of RNAse H. These experiments showed that extracts depleted in either U1, U2 or U4 snRNAs 3' cleaved and polyadenylated added precursor mRNAs as efficiently as the original extract (Berget and Robberson, 1986).

On the basis of the available evidence it would appear that the suggestion, made on theoretical grounds, that the U4 snRNA is involved in 3' processing is unlikely. However it does appear that an Sm-precipitable U snRNA is involved. This snRNA is probably not one of the common snRNAs already identified. It may be a snRNA that exists at low abundance similar to the U7 snRNA in sea urchin embryos. This snRNA might complex with proteins also present in other snRNPs such as the U1 snRNP.

It has been shown that *in vitro* the interaction between the conserved sequences, described previously, and the 3' processing machinery generates the mRNA 3' end by a 3' endonucleolytic cleavage. In the HeLa nuclear extract processing system it was shown that processing of adenovirus precursor mRNAs, at the L3 site, occurs (Moore and Sharp, 1985). In the absence of magnesium ions a small RNA species, representing the cut - off 3' extension from the precursor mRNA, was detected as well as the 3' processed RNA. This indicated that, in this system, 3' processing was achieved by an endonucleolytic cleavage at, or near, the mRNA 3' end position. The same result has been obtained using precursors to SV40 late mRNAs *in vitro* (Zarkower et al, 1986).

1.5 SMALL NUCLEAR RNAs

The small nuclear RNAs (snRNAs) are a class of small RNA molecules found in the eukaryotic cell nucleus, they can be divided into two classes based both on immunological characteristics and the type of RNA polymerase required for their transcription. These two classes are the U snRNAs and the La or class III snRNAs (for reviews see Ziaya, 1981, and Lerner and Steitz, 1981), of these it is the U snRNAs that have been the most studied. The U snRNAs are the snRNAs involved in mRNA processing reactions, they were so named because they are rich in U residues. The U snRNAs range in size from about 60 - 220 nucleotides. U1 and U2 snRNAs are the most abundant. U1 being present at about 1 x 10^6 copies per cell nucleus and U2 at about 5 x 10^5 copies per cell nucleus. U3 - U6 are present at 1 - 3 x 10^5 copies per cell. The series also includes the U7 snRNA, implicated in histone mRNA 3' processing (Galli et al, 1984). U7 is much less abundant than the U1 - U6 snRNAs, being present at an abundance 1/30 - 1/50 th of the molar concentration of U1 in sea urchin embryos (Strub et al, 1984, DeLorenzi et al,1986). There are also other low abundance snRNAs in the U RNA series. The genes coding for the U snRNAs have unusual promoters with no TATA homology at - 30 and no CAAT homology at - 70, - 80 (Cordan et al, 1980;

Bancist et al, 1980), they are transcribed by RNA polymerase II (Murphy et al, 1982) and the snRNAs made are not polyadenylated (Busch et al, 1982). The sequences and secondary structures of the snRNAs have been highly conserved in evolution. The snRNAs have an unusual 5' end in that U1 - U5 have a trimethylated cap structure while U6 has no identifiable cap, they also have a number of modified bases at the 5' end the significance of which is not known.

The U snRNAs are found in their active form associated with proteins in particles known as small nuclear ribonucleoprotein particles (snRNPs). It is these proteins that are the basis for the immunological precipitation of snRNAs. The snRNPs U1 - U6, are found in the cell nucleus. U3 is localised within the nucleus to the nucleolus. Antibodies that precipitate U snRNPs were first obtained from patients with the autoimmune disease serum lupus erythromatosus, a connective tissue disorder. One activity , anti - Sm, precipitates U1, U2, U4, U5, U6 and U7 snRNPs. Other activities, anti - U1 RNP and anti - U2 RNP, precipitate specifically U1 and U2 snRNPs. These antibodies have been extremely useful in determining the structure and function of the U snRNPs. Each snRNP contains about 7 - 8 proteins associated with one snRNA molecule. Of these proteins 5 form a common core in the Sm precipitable snRNPs. The other 2 - 3 proteins are snRNA specific and it is on these that the determinants recognised by anti - U1 RNP and anti - U2 RNP are found (Mimori et al, 1984; Petterson et al, 1984). An

exception to this rule appears to be the U4 and U6 snRNAs which are found in a single particle (Hashimoto and Steitz, 1984).

A region near the 3' end of the snRNA is important in the binding of the snRNP proteins (Mattaj and DeRobertis, 1985). For the U1, U2, U4 and U5 snRNAs the consensus A (U) n G (where n) 3) has been identified for this protein binding sequence. U6 lacks this sequence, presumably because it associates with U4 in a composite snRNP particle.

Fractionation and cross - linkage studies have shown that U1 - U6, with the exception of U3, are associated with heteronuclear RNA and U3 with ribosomal precursor RNAs, in the cell nucleus. (Hinterberger et al, 1983, Bringmann et al, 1983, Calvet et al, 1982). These associations correlate with evidence implicating the U snRNAs in mRNA splicing and 3' end formation.

The class III or La snRNAs have been less extensively studied. They range in size from 80 - 120 nucleotides and are not as highly conserved as the U snRNAs. They are precipitated by a class of antibodies called anti - La and they are transcribed by RNA polymerase III, their role within the cell is not clear.

The 3' processing of snRNAs

The U series of small nuclear RNAs are transcribed by RNA polymerase II to produce non polyadenylated transcripts. U1 and U2 snRNAs can be detected in the cytoplasm of HeLa cells as

short lived precursors. These precursors are 8 - 10 nucleotides longer at the 3' end than the final RNAs. Whilst in the cytoplasm the extra nucleotides are trimmed off (Elicairi and Sayavedra, 1976, Zieve and Penman, 1976, Madore et al, 1984). The precursors are precipitable by anti - snRNP anti - sera so this final processing reaction probably takes place after the snRNA has at least partially assembled into a snRNP particle. (Wieben et al, 1985). The sequences of the U snRNA genes have revealed several interesting features around the RNA 3' end position. A homology 3' to to the coding region of these genes has been identified (Zeller et al, 1984). This block consists of the sequence :

T T T N A A A G A A/T

Located between positions + 11 and + 23 in the Xenopus U1 snRNA gene and in a similar location in other snRNA genes (Zeller et al, 1984). It was suggested that this sequence is important in forming the 3' ends of snRNAs.

The human U2 gene contains this downstream consensus and it also has a terminal palindrome at the 3' end. When this gene was injected into Xenopus oocytes both a precursor RNA with a 10 nucleotide 3' extension and the mature RNA were detected. Deletion experiments showed that the 3' terminal palindrome was

not essential for 3' end formation however the downstream element was essential. (Yuo et al, 1985).

The human U1 snRNA gene also contains a 3' terminal palindrome and the downstream conserved sequence element. When transfected into HeLa cells this gene produced precursor RNAs with 2 - 3 extra nucleotides at the 3' end as well as the mature RNA. The 3' terminal palindrome was found not to be required for 3' end formation but the downstream element was necessary (Hernandez, 1985).

The Xenopus U7 genes also contain a similar conserved downstream sequence element (DeLorenzi et al, 1986).

The arrangement of the 3' ends of these genes is similar to the arrangement at the 3' ends of histone genes, there is a 3' terminal palindrome and an essential downstream conserved element. Intriguingly the sequence of the downstream sequence element has much in common with the sequence downstream of histone genes (see earlier in introduction). The requirement for these sequences for 3' processing is however slightly different between the two sets of genes.For histone genes the 3' terminal palindrome is necessary for mRNA 3' end formation whereas here it is not required. Despite this it is tempting to suggest that these two sets of genes might use related mechanisms for generating the RNA 3' end.

1.6 CHANGES IN RNA 3' PROCESSING DURING XENOPUS DEVELOPMENT ?

Oogenesis in Xenopus laevis is a long process lasting 6 months or more during which the oocyte is prepared genetically and physiologically for fertilisation. The process can be sub - divided into 6 stages (Dumont, 1972). Stage 1 oocytes are the earliest definable stage, they are small and transparent with their chromosomes at the zygotene, pachytene and diplotene stages of the first meiotic prophase. Stages 2 - 4 are vitellogenic stages during which the yolk is laid down, consequently they are opaque. Yolk deposition is over by stage 5. Stage 2 oocytes are also known as early lampbrush oocytes, stage 3 as maximal lampbrush oocytes and stage 4 as late lampbrush oocytes. During the lampbrush stages the chromosomes are extended with many loops, the sites of active transcription. Stage 6 oocytes are large fully developed oocytes with condensed chromosomes ready for the first meiotic metaphase. The oocytes mature in response to hormonal stimuli. During maturation the first meiotic division is completed, the germinal vesicle breaks down and the first polar body is expelled. The maturation response can be triggered *in vitro* by treating stage 6 oocytes with progesterone. Matured oocytes are laid as unfertilised eggs surrounded by a layer of jelly. On fertilisation the second meiotic division is completed to produce a haploid pronucleus which fuses with the sperm pronucleus. The resulting embryo then undergoes a period of rapid and synchronous cleavages during which there is no transcription (Newport and Kirschner, 1982a 1982b). At the mid - blastula

stage the cleavages become less synchronous and the transcription of many genes is switched on. Prior to the mid - blastula stage the embryo utilises a store of proteins accumulated during oogenesesis. It also makes it's own proteins by translating a maternal mRNA store also accumulated during oogenesis.

This strategy of early development raises several important questions; amongst them are what if any is the contribution of transcripts from the lampbrush chromosomes to the stored maternal mRNA pool and how is this pool activated after fertilisation ? In discussing these questions I will concentrate on the ways in which changes in 3' processing of mRNAs might be involved.

Classically the lampbrush chromosomes have been regarded as the site of production of the maternal mRNA pool (Davidson, 1976), this idea would seem reasonable as they are transcriptionally very active. We know that in the stage 6 oocyte 70% of the polyadenylated RNA contains repetitive sequences and these are not translatable (Anderson et al, 1982, Richter et al 1984), this RNA could be precursors to the mRNAs used in early cleavage. The presence of repeat sequences 3' of the transcript might be sufficient to prevent translation, these repeat sequences could be removed by a 3' cleavage reaction taking place after fertilisation.

The provision of histone proteins to the developing embryo has been investigated, this is achieved via the storage of histone protein in the oocyte and a increase in the translation of histone mRNA in the embryo compared to the oocyte. During oogenesis histone mRNA is translated at a low constant rate of 40

pg histone protein produced per hour to build up a protein store. When the oocyte matures this rate increases to about 2.5 ng per hour and at the maximal rate in the blastula embryo it is about 7 ng per hour (Adamson and Woodland, 1972). This translational activation is probably not due to the processing of histone precursor mRNAs made during the lampbrush stage. Firstly because histone transcripts are established at a peak level prior to the lampbrush stage and stay at a more or less constant level through the remainder of oogenesis (Van Dongen et al, 1981). Secondly there is no evidence to suggest histone precursor mRNAs exist in the stage 6 oocyte where they ought to be very abundant. Experiments described later in this thesis were performed to further investigate the latter point.

Studies have been performed into the accumulation of mRNAs, other than histone mRNAs, during oogenesis. Investigations of the accumulation of polyadenylated RNAs have been carried out by screening Northern blots of different stage oocyte and embryo RNAs with labelled cDNA clones from ovary and tadpole libraries (Golden et al, 1980, Colot and Rosbash, 1982). All 16 of the RNAs studied showed the same co - ordinate pattern of expression, they reached a peak level prior to the maximal lampbrush stage and remained more or less constant after that. In addition all the clones hybridised to a single band on the Northern's indicating that none was stored as a precursor for later processing. A more recent study (Dworkin and Dworkin-Rastl, 1985) investigated the changing titres of 90 sequences during oogenesis and maturation. Most were established in relative titre in pre - lampbrush

oocytes although some increased and several decreased during subsequent oogenesis. For some of the sequences non - adenylated transcripts longer than the corresponding adenylated transcripts were detected. The longer transcripts decreased in abundance in eggs compared to oocytes. These longer transcripts might have represented transcripts that had not undergone 3' processing and had been stored in a translationally inactive state. If these transcripts really were representatives of the stored maternal RNA pool they probably were not transcribed off the lampbrush chromosomes because they were established in titre prior to the maximal lampbrush stage.

An alternative way in which 3' processing might be involved in the translational activation of stored maternal mRNAs is through the control of the adenylation status of mRNAs. There is considerable evidence for complex, sequence specific polyadenylation changes during oogenesis, maturation and early embryogenesis. Histone mRNAs are an interesting example of sequences which change polyadenylation status on oocyte maturation (Ballantine and Woodland, 1985). Oocyte histone mRNAs have short poly - A tracts, of up to about 20 A residues ,at their 3' end. These tracts disappear on maturation. In somatic cells histone mRNAs are non - adenylated and they are efficiently transcribed. De - adenylation of histone mRNA on maturation correlates with an increase in translational activity.

More generally it has been shown that the initial response of oocytes to progesterone treatment is an increase, of 10 - 20 at, in the average poly - A length of transcripts and a 5 - 10%

increase in adenylated sequences. After germinal vesicle breakdown 35% or so of the adenylated sequences disappear (Darnborough and Ford, 1979).

These polyadenylation changes have been shown to be sequence specific, some sequences show an increase in adenylation on maturation whilst others show a decrease in adenylation (Colot and Rosbash, 1982).

On the basis of the available evidence it would seem that the lampbrush chromosomes are probably not responsible for the initial production of the store of oocyte RNA which forms the maternal mRNA pool, however transcription from the lampbrush chromosomes might play a part in replacing transcripts lost from the initial pool due to RNA turnover. Most of the sequences investigated are not stored as unprocessed precursors but this may happen in certain cases. Complex changes in the polyadenylation status of mRNAs occur during early development but these do not seem to follow any obvious rules.

1.7 PREVIOUS USE OF THE OOCYTE MICROINJECTION TECHNIQUE TO INVESTIGATE mRNA 3' PROCESSING

The stage 6 *Xenopus* oocyte is a very large (1 mm or more in diameter) single cell arrested in meiotic prophase. The nucleus in the oocyte is represented by a germinal vesicle bounded by a

nuclear membrane. Because of the large size of both the oocyte and its nucleus, components can be easily injected into either nuclear or cytoplasmic compartments and transcription or translation analysed. Early experiments showed that injected DNA can be replicated in unfertilised eggs and that mRNAs can be translated after injection into oocytes (Gurdon et al, 1969; Gurdon et al, 1971). It was soon established that the oocyte would transcribe many genes when the genes were injected into the nucleus, these included : SV40 DNA, adenovirus 5, Drosophila histones and øX174 (Mertz and Gurdon, 1977). Unfertilised eggs were also shown to transcribe injected DNAs, presumably because nuclear components are released on germinal vesicle breakdown. The amount of transcription on microinjection into eggs was less than on microinjection into the oocyte nucleus. Later work (DeRobertis and Mertz, 1977) demonstrated that mRNA transcribed in the oocyte, from injected, DNA was translated. As experiments became more sophisticated attempts were made to investigate the size of the transcripts and to map their termini. This was done using the sea urchin histone gene cluster h22 (Probst et al, 1979). Transcripts from the H2A and H2B genes were investigated. Transcripts made in the oocyte, off these 2 genes, were indistinguishable in size from transcripts made in the sea urchin. This result demonstrated that the oocyte has the mechanisms to form the 5' and 3' ends of certain heterologous mRNAs. A later investigation showed that for the H1, H2A and H2B genes from h22 the levels of correct 5' and 3' termini were about the same, whereas for the H3 gene the level of correct 3' termini

was much lower (Hentschel et al, 1980). When injected into unfertilised Xenopus eggs sea urchin histone genes again produced transcripts with correct termini (Bendig, 1981).

Many genes have now been injected into the oocyte nucleus and have been shown to produce transcripts with correct 3' termini. These genes include the chicken H2B (Krieg and Melton, 1984) and H5 (Wigley et al, 1985) genes both of which produce non - polyadenylated mRNAs in the oocyte. On injection of the Xenopus ß globin gene and the SV40 late transcription unit correctly 3' cleaved and polyadenylated mRNAs were produced (Mason et al, 1985, Wickens and Gurdon, 1983). Other genes do not produce correctly 3' cleaved and adenylated transcripts after injection into the oocyte nucleus, these include the human α globin gene and the bovine growth hormone gene (unpublished work cited in Conway and Wickens, 1985).

With the development of *in vitro* systems to make large quantities of defined RNA transcripts, artificial precursor mRNAs have been made and used to investigate 3' processing directly. This approach was used to show that the oocyte nucleus was capable of correctly splicing an artificial human ß globin precursor RNA . It was also claimed that accurate but inefficient 3' processing of these precursors could occur in the oocyte nucleus (Green et al, 1983).

Considerable success has been obtained using synthetic histone precursor RNAs in microinjection experiments. 3' processing has been demonstrated for a chicken H2B precursor mRNA (Krieg and Melton, 1984), a sea urchin H2A precursor mRNA and a

Xenopus H4 precursor mRNA (Georgiev et al, 1984). It has also been shown that a sea urchin H3 precursor mRNA was efficiently 3' processed in oocytes pre - injected with the U7 snRNA (Georgiev et al, 1985).

There have been no reports so far of artificial precursor mRNAs coding for adenylated non - histone mRNAs being 3' processed in the oocyte nucleus, except for the human ß globin precursor already mentioned.

1.8 PROJECT INTRODUCTION

In this thesis experiments in which microinjection into Xenopus oocytes, eggs and embryos was used to investigate mRNA 3' processing are described. The results are presented in two sections corresponding to two lines of work followed.

In section one histone mRNA 3' processing at different stages of development was investigated. The purpose of this work was to see whether changes in mRNA 3' processing could be correlated with the observed storage of maternal mRNAs in the oocyte and their utilisation in the embryo. Both 3' cleavage of precursor mRNAs and the polyadenylation status of the resulting mRNAs were considered. In these experiments artificial histone precursor mRNAs were made *in vitro*. These artificial precursor mRNAs resembled genuine histone precursor mRNAs in that they had extra downstream histone sequences at the 3' end. After microinjection of precursor mRNA into oocytes, eggs or embryos it was determined whether 3' cleavage and/or polyadenylation

reactions ocurred. The possible existance of stored histone precursor mRNAs in the oocyte was also investigated.

In section two the oocyte was used as an assay system to investigate the 3' processing of a polyadenylated mRNA. The gene mainly used in these studies was the mouse β (major) globin gene. This gene was chosen because it possesses all the features of a typical polyadenylated mRNA producing gene. In mouse erythroleukemia cells it had been shown that transcription terminated downstream of the mRNA 3' end position, an RNA 3' processing step was therefore likely to be necessary to form the 3' end of the mature mRNA (Hofer and Darnell, 1981; Hofer et al, 1982; Citron et al, 1984). When linked to a Xenopus histone promoter this gene was expressed in the oocyte nucleus. Cleavage and polyadenylation at the β globin mRNA 3' end position were then investigated. Artificial β globin precursor mRNAs were made, *in vitro*, and used in microinjection experiments with a view to identifying sequences involved in 3' processing. The Xenopus heat shock gene (hsp70A) was used in similar experiments because this was a Xenopus gene known to be expressed in the oocyte (Bienz and Gurdon, 1982).

2. MATERIALS AND METHODS

MATERIALS

Common laboratory chemicals were AnalaR grade and were purchased from BDH Chemicals Ltd, the Sigma Chemical Company Ltd, May and Baker Ltd and Fisons Scientific plc.

Restriction endonucleases and DNA modification enzymes

Restriction endonucleases were purchased from Amersham International plc, Bethesda Research Laboratories (U.K.), Boehringer - Mannheim and New England Biolabs, all were used according to manufacturers instructions.

T4 DNA ligase, T4 DNA polymerase , T4 polynucleotide kinase and E.coli poly A polymerase were purchased from Bethesda Research Laboratories.

E.coli DNA polymerase I (Klenow fragment), E.coli DNA polymerase I (Kornberg) and Calf intestinal alkaline phosphatase were purchased from Boehringer - Mannheim.

General enzymes

SP6 RNA polymerase and proteinase K were purchased from Boehringer - Mannheim.

S1 nuclease, lysozyme and RNase A were purchased from the Sigma Chemical Company.

Nucleotides and radioisotopes

Unlabelled dNTPs and rNTPs were purchased from Boehringer - Mannheim. The cap analogue $m^7G(5')ppp(5')G$ as a disodium salt was purchased from Pharmacia. All radioisotopes were purchased from Amersham International plc. α ^{32}P dGTP was of specific activity 2000 - 3000 Ci / mM at 10 uCi / ul in aqueous solution.

α ^{32}P rGTP was of specific activity 410 Ci / mM at 10 uCi / ul in aqueous solution.

γ ^{32}P rATP was of specific activity) 5000 Ci / mM at 10 uCi / ul in aqueous solution.

Reagents for electrophoresis

Agarose (type II) was purchased from Sigma, acrylamide was purchased from Fisons Scientific and bis - acrylamide was purchased from Eastman - Kodak.

Other reagents

Rnase inhibitor was purchased from Boehringer - Mannheim. oligo dT cellulose and MS222 (ethyl M - amino - benzoate) were purchased from Sigma. Saphadex G50 was purchased from Pharmacia and nitrocellulose manufactured by Schleicher and Schuell was purchased from Anderman and Co Ltd.

Xenopus laevis oocytes eggs and embryos.

Xenopus laevis oocytes, eggs and embryos were provided by the Warwick university Xenopus colony. This colony was stocked from commercial suppliers including Xenopus Ltd and the African Xenopus facility.

2.1 AGAROSE GEL ELECTROPHORESIS

Adapted from Maniatis et al, 1982.

The DNA to be electrophoresed was mixed with with agarose gel loading buffer in the ratio of 4 parts DNA solution to 1 part loading buffer.

Agarose gel loading buffer (10 ml)

3 ml glycerol

0.037 g EDTA (10 mM final concentration)

Orange G or bromophenol blue (enough to colour solution)

Made to 10 ml volume using TE buffer (10 mM Tris, 1mM EDTA, pH 8.0)

2 types of flat - bed agarose gels were used :

a. Mini - agarose gels

These gels were used where quick analysis was required and for gel isolation of DNA fragments. TBE buffer was used both to make and run the gels .

10 x TBE stock solution (1 litre)

108 g Trizma base

55 g boric acid

9.5 g disodium EDTA

pH adjusted to 8.3 using concentrated HCl

Routinely 1% gels were made using 0.5 g of agarose in 50 ml 1 x TBE buffer. Ethidium bromide was included in the gel at a

concentration of 1µg/ml. 50 ml of solution was required to make a gel 80 mm by 100 mm and about 5 mm in depth. Using a 8 well gel comb volumes of 20 - 25 µl could be loaded. The gel was run submerged in 1 x TBE buffer containing ethidium bromide at a concentration of 1 µg/ml. Mini - gels were run at 80 mA for about 30 minutes and DNA fragments visualised under U.V. light.

b. Large size agarose gels

These gels were used for better resolution of digests which contained many fragments of similar size and for gel isolation of DNA fragments from other similar sized fragments. The buffer used to make and run these gels was TAE :

20 x TAE stock solution (1 litre)	Concentrations in 1 x TAE
96.97 g Trizma base	40 mM
32.8 g anhydrous sodium acetate	20 mM
14.9 g disodium EDTA	2 mM

pH adjusted to 8.3 with glacial acetic acid.

Various concentrations of agarose were used depending on the size of fragments to be resolved. The agarose was melted in 150 ml of 1 x TAE and ethidium bromide to a concentration of 1 µg/ml was added. The gel was poured on a glass plate 195 mm by 150 mm in size. The gels were run at 120 Volts for 2 - 3 hours. The running buffer was 1 x TAE containing ethidium bromide. After electrophoresis DNA fragments were visualised under U.V. light.

2.2 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS

Adapted from Maniatis et al, 1982.

DNA fragments were isolated from agarose gels both for cloning and making pure probes for S1 nuclease analysis. The required fragment was visualised under U.V. light and a slot was made several mm in front of the DNA band using a razor blade. Two thicknesses of 3 MM filter paper the same depth as the gel and wide enough to take the whole band were inserted into the slot. The DNA band was then electrophoresed into the filter paper. This electrophoresis took 2 - 3 minutes; the migration of the band was checked periodically under U.V. light. The filter paper was then removed and transfered to a 0.75 ml eppendorf tube which had a pin - hole in the bottom. This tube was centrifuged for 20 seconds in a microfuge and the solution containing the isolated DNA fragment was collected in a 1.5 ml eppendorf tube. The filter paper was then washed a total of 3 times using TE buffer (10 mM Tris, 1 mM EDTA). The DNA solution from each wash was collected in a 1.5 ml eppendorf tube. After the final wash the DNA solutions were pooled and sodium acetate to a final concentration of 0.3 M was added. The DNA solution was extracted twice with phenol and once with chloroform, after which the DNA was collected by ethanol precipitation.

2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

Based on Maniatis et al, 1982.

This technique was used in a number of different procedures as outlined below :

Procedure	gel type	conditions
Gel isolation of DNA fragments for S1 hybridisation probes or primers	6%	denaturing
Gel isolation of DNA fragments for Maxam and Gilbert sequencing	6%	non - denaturing
S1 nuclease analysis	6% / 8%	denaturing
Maxam and Gilbert sequencing	6% / 20%	denaturing
Analysis of labelled RNA from oocyte and embryo injections	6% standard or thick gel	non - denaturing

These gels were made and electrophoresed using TBE buffer. Gel mixtures were made up as follows :

	6%		8%	20%
	standard	"thick"		
Urea	18.9 g	37.8 g	18.9 g	18.9 g
Acrylamide	2.7 g	5.4 g	3.6 g	9.0 g
bis - acrylamide	0.135 g	0.27 g	0.18 g	0.45 g
10 x TBE	4.5 ml	9.0 ml	4.5 ml	4.5 ml
Final volume (made up with distilled water)	44.5 ml	89.0 ml	44.5 ml	44.5 ml
To polymerise :				
10% ammonium persulphate	300 µl	600 µl	300 µl	300 µl
TEMED	36 µl	72 µl	36 µl	36 µl

Standard gels were poured using plates 385 mm by 200 mm with 0.4 mm thick spacers. Thick gels were poured in the same size gel plates using 1 mm thick spacers. Non- denaturing gels were made as above but urea was not included in the mix. Samples were resuspended in 2 - 3 µl of acrylamide gel loading buffer for standard gels and up to 15 µl of loading buffer for thick gels.

Acrylamide gel loading buffer

1 x TBE buffer

20% glycerol

Bromophenol - blue (sufficient to colour solution)

Samples for denaturing gels were heated to 95°C for 5 minutes immediately prior to loading. Samples for non - denaturing gels

were not heated. Electrophoresis was carried out in a vertical gel tank. TBE buffer was used in the upper and lower reservoirs. Standard gels were electrophoresed at 22mA, thick gels were electrophoresed at 18 mA.

After electrophoresis standard gels were covered with "cling" film and autoradiographed at -70°C with an intensifying screen. Thick gels were fixed in 10% glacial acetic acid for 30 minutes and dried down onto 3 MM filter paper before autoradiography.

2.4 ISOLATION OF DNA FRAGMENTS FROM POLYACRYLAMIDE GELS

Based on Maxam and Gilbert, 1980.

Isolation of radioactively labelled DNA fragments from acrylamide gels was used both in the production of pure S1 probes, where 2 DNA fragments were too similar in size to be separated on an agarose gel, and in the preparation of DNA for Maxam and Gilbert sequencing. The method used worked efficiently for fragments under about 800 nt in size.

The DNA restiction fragments were radioactively labelled and electrophoresed on a denaturing or non - denaturing acrylamide gel, depending on the future use of the isolated fragment. After electrophoresis the gel was covered with "cling" film and 2 strips of 3 MM filter paper were taped on top. Radioactive ink

spots were then applied to the 3 MM paper in an asymmetric pattern, these spots were used later to orientate the autoradiograph. The gel was autoradiographed for 5 - 10 minutes at room temperature. The autoradiograph was developed and the DNA band to be isolated was located on the X - ray film. The band was cut out of the X - ray film to provide a template for cutting the band out of the acrylamide. The X - ray film was positioned on the gel and orientated using the radioactive ink spots. The acrylamide gel slice was then cut from the gel. The cut gel slice was transfered to a 1.5 ml eppendorf containing 330 µl of polyacrylamide gel elution buffer, this was incubated overnight at 37°C. The gel slice was then removed from the buffer and the DNA solution was phenol extracted twice. The labelled DNA was recovered by ethanol precipitation.

Polyacrylamide gel elution buffer

0.5 M ammonium acetate

0.01 M magnesium acetate

0.1 % SDS

0.1 mM EDTA

2.5 CLONING IN SP6 PLASMIDS

For a detailed description of the SP6 sub - clones made see materials and methods sections 2.26 and 2.27. Described here are the general methods used in making SP6 sub - clones. The SP6 plasmids pSP64 and pSP65 (Melton et al, 1984) contain the SP6 promoter followed by a polylinker sequence. The polylinker sequence is in opposite orientations in the 2 plasmids (see figure 1). To be used for *in vitro* transcription the target sequence needs to be cloned into the polylinker downstream of the SP6 promoter. The orientation of the insert determines whether the transcripts made are sense or anti - sense.

a. Preparation of vector DNA

1 ug of SP6 plasmid DNA was restricted with one or more of the polylinker enzymes. Half of the digest was electrophoresed on an agarose gel to check that the digest was complete. The remainder of the digest was phenol extracted, the DNA recovered by ethanol precipitation and resuspended in 20 µl sterile water. The linearised vector DNA was then treated with calf intestinal alkaline phosphatase (CIP) to remove 5' phosphate groups.

Vector DNA solution	20.0 µl
10 % SDS	0.5 µl
1M Tris pH 8	1.0 µl
CIP	2.0 µl (1 unit)
Water	26.5 µl

The reaction was incubated at 37°C for 30 minutes, a further 2.0 µl of CIP was then added and the reaction incubated for a further 30 minutes at 37°C. 4 ul of 10 % SDS was added and the reaction mix heated at 68°C for 15 minutes. 6 ul of 3M sodium acetate was added and the reaction mix was phenol extracted once, chloroform extracted once and ether extracted once. The vector DNA was recovered by ethanol precipitation and resuspended in 20 µl of sterile water.

b. Preparation of target DNA

This was usually prepared by restriction enzyme digestion followed by isolation of the required fragment from an agarose gel. In some cases this was not possible and a mixture of fragments were cloned, clones containing the required fragment were identified at a later stage. The target DNA was ethanol precipitated and resuspended in water to give about 10 ng DNA / ul.

c. Ligations

These were set up as follows :

Linear, CIPed pSP64 or pSP65 DNA	1 ul (about 20 ng)
Target DNA	5 ul (about 50 ng)
10 mM ATP	1 ul
10 x C buffer	1 ul
T4 DNA ligase	1 ul (2 - 4 units)
sterile water	1 ul

As controls ligations were also set up containing CIPed vector alone and target DNA alone. The ligations were incubated overnight at 4°C.

10 x C buffer

500 mM Tris / Cl pH 7.6

100 mM magnesium chloride

200 mM dithiothreitol

d. Transformations

For Transformation an active plate of E.coli strain DH1 was required. This strain is ampicillin sensitive. The SP6 plasmid confers ampicillin resistance so this was the selectable marker used. An ampicillin sensitive colony of DH1 was picked and grown in a 10 ml aliquot of L broth for 3 - 5 hours until the O.D. at 590 nm reached 0.3. Competent cells were then prepared. The 10 ml culture was centrifuged in a MSE bench centrifuge at top speed for 10 minutes. The pelleted cells were resuspended in 5 ml of ice cold 0.1 M magnesium chloride. The cells were recentrifuged and resuspended in 5 ml of ice cold 0.1 M calcium chloride. The cells were recentrifuged and resuspended in 500 ul of ice cold 0.1 M calcium chloride, they were then incubated on ice for 90 minutes. The competent cells were divided into 5 x 100 ul aliquots. One 100 ul aliquot was required for each transformation so 1 x 10 ml culture of DH1 gave sufficient cells for 5 transformations. The ligation mix was added to the 100 ul aliquot of competent cells, they were then left on ice for 30 minutes for transformation to occur. The transformed cells were heat shocked

at 42°C for 2 minutes and incubated on ice for a further 15 minutes. The cells were then plated out on ampicillin plates. The cells were split into 90 ul and 10 ul aliqouts which were plated onto separate plates. This gave 2 different colony densities. The plates were incubated overnight at 37°C and examined. Usually the CIPed vestor ligated without target DNA gave very few transformants indicating that the CIP reaction was efficient. The number of colonies on the plates where target DNA was included varied, typical numbers were about 20 from the 10 ul aliquot and 150 from the 90 ul aliqout.

L broth (500 ml)

Difco tryptone 5.0 g
Yeast extract 2.5 g
sodium chloride 5.0 g
Autoclaved before use

L agar (500 ml)

To make L agar 7.5 g of Bacto agar was weighed out and 500 ml L broth was added. After autoclaving this was sufficient to make about 18 standard size plates (90 mM diameter).

Ampicillin plates

An ampicillin stock solution at a concentration of 10 mg/ml ampicillin was made. 5.0 ml of this stock was then added to 500 ml of autoclaved L agar. This gave a final ampicillin concentration of 100 ug / ml. plates were then poured in the usual way. Both L agar and ampicillin plates were dried for about 30 minutes before use.

e. Screening transformants

This was done by making small scale plasmid preparations (mini - preps) from a number of colonies and carrying out digests on the

plasmid DNAs. The method used for these mini - preparations was based on the alkaline lysis method (Birnboim and Doldy, 1979) and is outlined below :

Initially 8 colonies were picked from the transformed plate. Each was grown up in L broth containing ampicillin (100 ug/ml) for 5 - 6 hours. The bacteria were then pelleted by centrifugation and each pellet was resuspended in 1 ml of the L broth supernatant. At this point glycerol stocks of the bacteria were made. To make the stock 200 ul of the resuspended bacteria were mixed with an equal volume of sterile glycerol. The glycerol stocks were stored at - 20°C. The remaining 800 ul of each culture was transfered to a 1.5 ml eppendorf tube and centrifuged for 3 minutes in an eppendorf microfuge. The supernatant was poured off and each pellet was resuspended in 120 ul of the following solution :

25 mM Tris / Cl pH 8

10 mM EDTA

15 % sucrose

2 mg / ml lysozyme

The cells were incubated on ice for 10 minutes then 240 ul of the following solution was added :

0.2 mM sodium hydroxide

1 % SDS

The DNA preparations were then left on ice for a further 5 minutes. 150 ul of 3M sodium acetate pH 4.8 was then added and the preparations left on ice for a further 5 minutes. The preparations were then centrifuged for 5 minutes in a microfuge

and the supernatants containing the plasmid DNA were removed. The supernatants were phenol extracted twice and chloroform extracted once before ethanol precipitation. Each DNA pellet was resuspended in 20 ul of water and used in restriction enzyme digests. Each mini - preparation yielded enough plasmid DNA for 4 - 5 digests. Because the preparations were not particularly pure an excess of restriction enzyme was used in these digests. The preparations also contained large amounts of RNA, for this reason 2 ul of RNase A (20 mg / ml stock) was added to each 20 ul restriction digest at the end of the digest time. The preparations were incubated for a further 10 minutes at 37°C in the presence of RNase. For most sub - clones it was possible to tell which preparations contained the required insert in the required orientation from 1 or 2 restriction digests.

2.6 LARGE SCALE PLASMID PREPARATION

A 10 ml culture of L broth containing ampicillin (100 ug / ml) was innoculated with 50 ul of bacterial glycerol stock from the mini - preparation. This 10 ml culture was grown overnight with shaking at 37°C. The 10 ml culture was then used to innoculate 500 ml of L broth containing ampicillin (100 ug / ml). The 500 ml culture was grown, shaking at 37°C, until the O.D. 540 nm reached 0.8, this took about 4 hours. 50 mg of chloramphenicol (final concentration 100 ug / ml) was added and the culture grown, shaking overnight at 37°C. The bacteria were pelleted by centrifugation at 6000 RPM for 15 minutes at 4°C using an MSE MS18 centrifuge. The 500 ml culture was centrifuged in 2 x 250 ml bottles so 2 bacterial pellets were obtained from one culture.

Each pellet was resuspended in 12 ml of 10% sucrose ; 50 mM Tris pH 8.0. The bacteria were transfered to a 50 ml tube and 2.5 ml of a 10 mg / ml lysozyme solution was added. The tube was then incubated on ice for 10 minutes. 2.5 ml of 0.4 M EDTA pH 8.0 was then added and the tube incubated on ice for a further 10 minutes. 20 ml of triton lysis solution was added and after mixing the tube was incubated on ice for 5 minutes.

Triton lysis solution

50 mM EDTA pH 8.0

50 mM Tris / Cl pH 8.0

0.1 % Triton X - 100

The bacterial lysate was cleared by centrifugation at 16000 RPM for 60 minutes at 4°C. After centrifugation the supernatant was poured off and divided between 2 X 50 ml tubes. The DNA solution was phenol extracted 2 X, chloroform extracted 1 X and dialysed for 2 x 2 hours, each dialysis was against 2 litres of TE buffer and was performed at 4°C. The volume of the DNA solution after dialysis was measured and sodium chloride was added to 0.3 M. The DNA was then precipitated. The DNA precipitate was collected by centrifugation at 8000 RPM for 30 minutes at 4°C. The ethanol was drained off and each pellet was resuspended in 10 ml of 1/10 th SSC buffer.

1 x SSC Buffer

0.15 M sodium chloride

0.015 M sodium citrate

30 ul of RNase A (20 mg / ml stock) was added to each 10 ml of plasmid DNA. The plasmid DNA was treated with RNase for 1 hour at

37°C. The DNA was then phenol extracted, chloroform extracted and ethanol precipitated. The precipitate was collected by centrifugation at 10000 RPM for 20 minutes. The ethanol was drained and the DNA pellet dried. The total yield of DNA from one 500 ml culture was resuspended in 10 ml 1/10 th SSC for loading on a caesium chloride gradient. Caesium gradients were set up in Beckman heat seal centrifuge tubes. 52 g of caesium chloride was dissolved in 40 ml 1 / 10 th SSC. 25 ml of this solution was put into each of 2 tubes. 5 ml of the plasmid DNA and 1 ml of ethidium bromide (10 mg / ml stock) were also added. The tubes were balanced and the gradients covered with liquid paraffin before the tubes were sealed. Centrifugation was at 45000 RPM and 18°C in the VTi 50 rotor in a Beckman L8 centrifuge. The gradients were centrifuged for 16 hours or greater before extraction of the supercoiled plasmid DNA band. The plasmid DNA from the gradients was extracted with isoamyl alcohol to remove the ethidium bromide. 5 or 6 extractions with an equal volume of isoamyl alcohol were necessary. The isoamyl alcohol was removed by dialysing the DNA solution against 2 x 2 litres of TE buffer, at 4°C, for a total of about 24 hours. The DNA solution was made 0.3 M with sodium chloride and the DNA was recovered by ethanol precipitation. The final pellet was resuspended in sterile water. A sample was removed and diluted to allow the DNA concentration of the preparation to be calculated from the absorbance at 260 nm. The plasmid DNA solution was diluted to a convenient working concentration about 1 mg / ml.

2.7 SP6 TRANSCRIPTION OF CLONED DNAs

Adapted from Green et al,1983.

Plasmids containing an insert downstream from the SP6 promoter were linearised using a restriction enzyme that cut in the SP6 polylinker downstream of the insert. On transcription with SP6 polymerase, in the presence of a radioactively labelled nucleotide, run - off transcripts of high specific radioactivity were generated for microinjection experiments. 2 ug of SP6 plasmid DNA was digested with the required restriction enzyme. The digest was stopped by phenol extraction and the linear DNA was recovered by ethanol precipitation. The DNA was resuspended in 2 ul of sterile water. 1 ul was electrophoresed on an agarose mini - gel to check that the digest was complete. The remaining 1 ug of DNA was used in the SP6 transcription reaction. The SP6 transcription mix was set up at room temperature by the addition of components in the following order:

Linear DNA	1 ul (1 ug)
low [G] 5 x rNTP mix	4 ul
α ^{32}P rGTP	2 ul (20 uCi)
200 mM dTT	1 ul
RNase inhibitor	1 ul (30 units)
5 mM cap analogue	4 ul
5 x transcription buffer	4 ul
1 mg / ml BSA	2 ul
SP6 polymerase	1 ul (10 units)

Low I. G 1.5 x rNTP mix	
25 mM rCTP	25 ul
25 mM rATP	25 ul
25 mM rUTP	25 ul
25 mM rGTP	1 ul
water	174 ul

5 x transcription buffer	
200 mM Tris / Cl pH 7.5	
30 mM magnesium chloride	
10 mM spermidine	

The final concentration of the 3 unlabelled rNTPs in the transcription mix was 500 uM, the final concentration of rGTP was 20 uM. The cap analogue $m^7G(5')ppp(5')G$ as a disodium salt was included in the mix to ensure that the transcripts were predominantly capped. The transcription was carried out at 41°C for 1 hour. After incubation the reaction mix was treated with RNase - free DNase I for 10 minutes at 37°C. The final concentration of DNase I used was 20 ug / ml. At this point a sample from the transcription was taken and dotted onto DE81 paper. This sample was used, along with a second sample taken later, to calculate the percentage incorporation of labelled nucleotide and the yield of SP6 RNA. 2 ul of 0.4 M EDTA, 2 ul of 1 ug / ul carrier tRNA and 50 ul of water were added to the mix. The solution was phenol extracted twice and chloroform extracted once before being ammonium acetate precipitated twice. Ammonium acetate precipitation was used to recover the SP6 RNA because this precipitation is more efficient than standard salt / ethanol precipitations at separating the SP6 RNA from unincorporated nucleotides. Ammonium acetate was added to 0.7 M followed by 3 volumnes of ethanol. Precipitation was either on dry ice or at -

20°C. The SP6 RNA was dried under vacuum and resuspended in water for injection. The amount of water used depended on the yield of SP6 RNA. For a typical reaction the volume was 4 - 6 ul which gave an RNA concentration of about 100 ug / ml.

Calculation of yield from SP6 transcription

To calculate the yield of SP6 RNA from a transcription it was necessary to work out the percentage incorporation of labelled rGTP. This was done by comparing the total counts in the transcription mix with the counts incorporated into SP6 RNA. To obtain total counts a 0.5 ul sample from the reaction mixture after treatment with RNase was taken (total volume 21 ul). This aliquot was diluted 1 + 9 and 0.5 ul aliquots of the diluted sample were spotted onto 4 identical squares of DE81 paper. These squares were counted directly. To obtain incorporated counts the SP6 RNA after the first ammonium acetate precipitation was resuspended in 20.5 ul of water. 0.5 ul of this was removed, diluted 1 + 9 and spotted onto 4 squares of DE81 paper. The 4 squares of DE81 paper for the incorporated counts were washed 5 times. Each wash was for 2 minutes in 200 ml of $Na_2HPO_4(H_2O)_{12}$ at a concentration of 50g / litre. After a final rinse in tap water the squares were dried and counted. The 4 readings of total counts and the 4 readings of incorporated counts were averaged and used to calculate the percentage incorporation of labelled nucleotide. The incorporation was usually about 75%. The yield of RNA from the SP6 transcription was then calculated by multiplying the percentage incorporation of labelled nucleotide

by the maximum theoretical yield from the transcription. Under the conditions used for transcription it was calculated that 75% incorporation of labelled nucleotide indicated that about 430 ng of SP6 RNA had been produced. In the microinjection experiment 2 ng RNA in 20 nl of solution was injected. The RNA was therefore required at a concentration of 100 ug / ml. To achieve this the 430 ng of SP6 RNA was resuspended in about 4.3 ul of water for injection

2.8 MAXAM AND GILBERT DNA SEQUENCING

Based on the method described in Maxam and Gilbert, 1980.

About 1 ug of DNA was restricted at the restriction site from where the sequence was required . The DNA was then radioactively end - labelled using either Klenow polymerase and α ^{32}P dGTP or T4 Kinase and γ ^{32}P ATP depending on the sequence of the restriction site. After labelling and removal of the modification enzyme the labelled DNA was restricted a second time in order to generate a DNA fragment labelled only at one end for sequencing. This labelled fragment was isolated from an acrylamide gel and the DNA eluted overnight at 37°C. The sequencing reactions were then performed. The isolated labelled DNA fragment was ethanol precipitated from the polyacrylamide gel elution buffer after the addition of 5 ug of carrier tRNA. The DNA was resuspended in 90 µl of water and 10 µl of 3 M sodium acetate was added. 20 µl of this solution was put into each of 5 x 1.5 ml eppendorf tubes and the DNA re - precipitated. Each DNA pellet was washed 3 times

with 80% ethanol to remove salt. The pellets were then dried under vacuum.

a. G reaction

The DNA pellet was dissolved in 200 ul of 50 mM sodium cacodylate, 1 mM EDTA pH 8.0. 1 ul of dimethyl sulphate was added and after mixing the reaction was incubated at room temperature for 2 ½ minutes. The reaction was stopped by the addition of 50 ul of G stop solution.

G stop solution

1.5 M sodium acetate pH 7

1.0 M β mercaptoethanol

100 ug / ml E.coli tRNA

750 ul of ethanol was added and the DNA was precipitated. The pellet was resuspended in 200 ul of water, after the addition of sodium acetate to 0.3 M it was reprecipitated. The pellet was washed with 80% ethanol and dried under vacuum.

b. A > C reaction

The DNA was dissolved in 100 ul of 1.2 M sodium hydroxide, 1 mM EDTA and incubated at 90°C for 9 minutes. The reaction was stopped by the addition of 150 ul 1 M acetic acid. 5 ug of carrier th~4 was added and the DNA recovered by ethanol precipitation as in the G reaction.

c. C reaction

The DNA was dissolved in 5 ul of water and 15 ul of 5M sodium chloride followed by 30 ul of hydrazine were added. The reaction was incubated at room temperature for 7½ minutes then stopped by the addition of 200 ul of C stop solution.

C stop solution

0.3 M sodium acetate

0.1 mM EDTA

25 ug / ml carrier tRNA

The DNA was recovered by ethanol precipitation as previously described.

d. C + T reaction

The DNA was dissolved in 20 ul of water. 30 ul of hydrazine was then added and the reaction incubated at room temperature for 7½ minutes. The reaction was stopped by the addition of 200 ul C stop solution and the DNA recovered by ethanol precipitation as previously described.

e. A + G reaction

The DNA was dissolved in 6 ul of water. 25 ul of formic acid was added and the reaction incubated at room temperature for 4 minutes. The reaction was stopped by the addition of 200 ul of 1 M sodium acetate containing 5 ug / ml carrier tRNA. The DNA was recovered by ethanol precipitation as described above.

The cleavage reaction was then carried out on the base - modified DNA. A piperidine stock was diluted 1 + 9 with water to make it approximately a 1 M solution. Each washed and dried DNA pellet was resuspended in 100 ul of 1 M piperidine. The reactions were then incubated at 90°C for 30 minutes. At the end of the incubation each reaction mix was frozen using dry ice and the piperidine removed by freeze drying the samples for about 3 hours. The dried samples were resuspended in 100 ul of water and

freeze dried again, this time overnight. Finally these dried samples were resuspended in 20 ul of water and freeze dried for a further 2 hours. The samples were resuspended in 4 ul acrylamide gel loading buffer and split into 2 x 2 ul aliquots. These samples were heated to 95°C for 5 minutes before loading onto an acrylamide sequencing gel. The gel was either 8% or 20% depending on the number of bases to be resolved. 2 runs of different time length were carried out on a single acrylamide gel using 1 x 2 ul aliquot of DNA from each reaction for each run. The samples were loaded in the following order :

G A + G A > C C C + T

After running at 26 mA the gel was covered with "cling" film and autoradiographed at - 70°C.

2.9 END - LABELLING DNA FRAGMENTS

End - labelled DNA probes were required for S1 nuclease analysis. In this technique it was possible to use a mixed probe containing a number of labelled DNA fragments, however it was preferable to use a pure probe consisting of only one end - labelled DNA fragment. The fragment to be labelled was isolated from an agarose gel and one of the following labelling methods was used : 3' end labelled probes were used to map the 3' ends of transcripts. 2 methods were used to make these probes.

a. 3' end - labelling using Klenow polymerase

Adapted from Maniatis et al, 1982.

100 - 500 ng of DNA was ethanol precipitated and after drying it was resuspended in 10 ul of Klenow buffer.

Klenow buffer

10 mM Tris / Cl pH 7.4

50 mM sodium chloride

10 mM magnesium chloride

Klenow polymerase was used to fill in 5' overhangs. A labelled nucleotide was included in the reaction mix to be incorporated opposite a complementary nucleotide in the template strand. α ^{32}P dGTP was usually used as the label. For some restriction sites where dGTP was not the first nucleotide to be filled in unlabelled nucleotides were also included in the reaction.

Reaction mixture :

DNA	10 ul (in Klenow buffer)
2 mM dNTP	1 ul (included if necessary)
α ^{32}P dGTP	2 ul
Klenow polymerase	2 ul (2 - 4 units)

The reaction was incubated for 40 minutes at room temperature then stopped by phenol extraction. The mixture was run down a sephadex G50 column and the excluded peak collected. Carrier tRNA was added and the sample phenol extracted twice and chloroform extracted once before ethanol precipitation.

b. 3' end labelling using T4 polymerase

Adapted from Maniatis et al, 1982.

Certain restriction enzyme sites cannot be 3' end - labelled using Klenow polymerase and labelled dGTP, for these restriction sites an alternative method of labelling using T4 DNA polymerase was used. T4 polymerase has a 3' to 5' exonuclease activity in addition to a 5' to 3' polymerase activity. If T4 polymerase is

added to a DNA fragment. in the presence of one labelled nucleotide, the enzyme will degrade the DNA strands in the 3' to 5' direction until it reaches a nucleotide identical to the labelled nucleotide, it will then continually remove and replace this nucleotide. In this way the labelled nucleotide is incorporated into the DNA. Approximately 500 ng of DNA was resuspended in 10 ul TA buffer. 2 ul of α ^{32}P dGTP and 1 ul of T4 DNA polymerase (containing about 0.25 units) was added. The reaction was incubated at 37°C for 40 minutes. The reaction was stopped by phenol extraction and the labelled DNA was separated from the unincorporated nucleotides using a Sephadex G50 column.

10 x TA buffer

0.33 M Tris acetate pH 7.9

0.66 M potassium acetate

0.1 M magnesium acetate

1 mg / ml bovine serum albumin

5 mM dithiothreitol

c. 5' end - labelling using T4 polynucleotide kinase

Adapted from Maniatis et al, 1982.

100 - 500 ng of the DNA fragment to be labelled was prepared by gel isolation from an agarose gel, this DNA was resuspended in 10 ul of water. The DNA was then treated with calf intestinal alkaline phosphatase (CIP) to remove the 5' phosphate groups.

10 ul DNA (100 - 500 ng)

5 ul 10 x CIP buffer

4 ul CIP (2 units)

31 ul sterile water

The reaction was incubated at 37°C for 30 minutes, a further 4 ul of CIP (2 units) was then added and the reaction incubated for a further 30 minutes. 5 ul of 10% SDS, 35 ul of water and 10 ul of 3 M sodium acetate were added and the reaction incubated at 68°C for 15 minutes to destroy the CIP. The CIPed DNA was phenol extracted 2 x, chloroform extracted 1 x and ether extracted 1 x, it was then recovered by ethanol precipitation. The DNA pellet was resuspended in 10 ul of water and added to the following reaction :

10 ul CIPed DNA
2 ul 10 x kinase buffer
0.5 ul 1M dithiothreitol
4 ul γ ^{32}P ATP
2 ul T4 polynucleotide kinase (10 - 15 units)
1.5 ul water

10 x kinase buffer

0.7 M Tris / Cl pH 7.6
100 mM magnesium chloride
50 mM dithiothreitol

The reaction was incubated at 37°C for 30 - 45 minutes. 2 ul of 0.4 M EDTA was then added and the reaction phenol extracted. The aqueous phase from the phenol extraction was passed down a sephadex G50 column to separate the labelled DNA from unincorporated nucleotides. The fractions containing the labelled DNA were phenol extracted 2 x, chloroform extracted 1 x and the labelled DNA was recovered by ethanol precipitation.

2.10 LABELLING DNA BY NICK - TRANSLATION

Based on Rigby et al, 1977.

This method of labelling DNA was used to make hybridisation probes for use on Northern blots.

The DNA fragment to be labelled was prepared by gel isolation from an agarose gel. Approximately 100 ng of DNA was resuspended in 9 µl water, this solution was added to the following mix :

4 µl 5 x nick translation buffer

1 µl 0.2 mM dATP

1 µl 0.2 mM dTTP

1 µl 0.2 mM dCTP

2 µl α ^{32}P dGTP

1 µl DNase I (final concentration 20 ng / ml)

9 µl DNA solution

This mix was incubated at 37°C for 15 minutes to introduce nicks into the DNA. 1.5 µl (2 - 5 units) of *E.coli* DNA polymerase I (Kornberg) was added and the mixture incubated at 16°C for 3 hours. The reaction was stopped by phenol extraction. The reaction mixture was then run down a sephadex G50 column to separate the labelled DNA fragment from the unincorporated nucleotides. The labelled DNA was collected by ethanol precipitation.

5 x nick translation buffer

0.25 M Tris / Cl pH 7.2

0.05 M magnesium sulphate

0.5 mM dithiothreitol

250 ug / ml bovine serum albumin

(stored in aliquots at - 20°C)

2.11 RADIOACTIVELY LABELLED DNA SIZE MARKERS

Radioactively labelled DNA fragments of known size were used as size markers for polyacrylamide gel electrophoresis. These were made by labelling restriction enzyme digests of pBR322 (Bolivar et al, 1977) or pAT153 (Twigg and Sherratt, 1980) using α ^{32}P dGTP. The restriction digests used to make markers and the sizes (in nucleotides) of the DNA fragments generated are detailed below :

pAT153 HinF I	pBR322 HinF I	pBR322 Hpa II	
1631	1631	622	180
517	517	527	160
396	506	404	147
298	396	309	122
221	344	242	110
220	298	238	90
154	221	217	76
145	220	201	67
75	154	190	34
	75		26
			15
			9

The DNA fragments were briefly treated with T4 DNA polymerase I in the absence of added nucleotides. During this period the exonuclease activity of the enzyme "nibbled back" in the 3' to 5' direction. A mix containing all 4 dNTPs, including 1 labelled nucleotide, was then added allowing the polymerase activity of the enzyme to polymerise in a 5' to 3' direction incorporating the labelled nucleotide. 1 ug of DNA was restricted with the required enzyme. Half of the digest was electrophoresed on a 1% agarose mini - gel to check for complete digestion. The remainder was ethanol precipitated and resuspended in 10 µl of TA buffer.

10 x TA buffer

0.33 M Tris acetate pH 7.9

0.66 M potassium acetate

0.1 M magnesium acetate

1 mg / ml bovine serum albumin

5 mM dithiothreitol

1 unit of T4 DNA polymerase was added and the reaction incubated at 37°C for 3 minutes. 4 µl of a mix containing all 4 dNTPs, including a radioactively labelled dNTP, was then added and the reaction incubated for a further 40 minutes at 37°C.

dNTP mix

1 µl 2 mM dATP

1 µl 2 mM dTTP

1 µl 2 mM dCTP

1 µl α ^{32}P dGTP

The reaction was stopped by phenol extraction and the reaction mixture was put down a sephadex G50 column. The excluded peak containing the labelled DNA fragments was collected. The marker was ethanol precipitated and resuspended in 50 - 100 µl of water. Before use on acrylamide gels 0.5 - 1.0 µl of marker was added to 3 µl of acrylamide gel loading buffer and heated to 95°C for 5 minutes.

2.12 S1 NUCLEASE ANALYSIS

Based on Berk and Sharp, 1977.

This technique was used to map the 3' ends of transcripts made in oocytes from injected genes. A 3' end - labelled DNA probe was prepared by labelling a gel isolated DNA fragment. Klenow polymerase was usually the enzyme of choice for this end - labelling reaction. The amount of probe used per S1 hybridisation depended on the specific radioactivity of the probe. Typically about 200 ng of the DNA fragment was labelled and used for about 10 S1 hybridisations. The amount of probe used per S1 reaction was not critical as long as the probe was in excess over the transcript to be mapped. The probe was resuspended in S1 hybridisation buffer at a volume of 5 ul of buffer for each S1 hybridisation. The RNA from injected and control oocytes was resuspended in S1 hybridisation buffer using 5 ul of buffer per oocyte equivalent of RNA.

S1 hybridisation buffer

40.0 mM PIPES pH 6.4

1.0 mM EDTA

0.4 M sodium chloride

80 % formamide

pH adjusted to 6.4 with concentrated sodium hydroxide.

Buffer stored at - 20°C

S1 hybridisations were set up in 1.5 ml eppendorf tubes using 10 ul of RNA and 5 ul of probe. After mixing each hybridisation mix

was run into a drawn glass capillary, these were then sealed. The capillary tubes were heated at 80°C for 7 minutes to denature the probe, they were then quickly transfered to the required hybridisation temperature. For some experiments the optimum hybridisation temperature was determined by using a range of different hybridisation temperatures in a series of test reactions. In other experiments a temperature of 55°C was used as this was found to be a satisfactory working temperature for most probes. Hybridisations were usually left for 3 hours but occaisonally overnight hybridisations were carried out with no loss of sensitivity. After hybridisation each mix was expelled into 150 ul of ice cold S1 nuclease buffer containing 225 units of S1 nuclease. The hybrids were treated with S1 nuclease for 40 minutes at 37°C. Each S1 nuclease reaction was then stopped by the addition of 3 ul of 0.4 M EDTA followed by phenol extraction. The hybrids were recovered by ethanol precipitation and dried before being resuspended in 2 ul of polyacrylamide gel loading buffer.

S1 nuclease buffer

0.28 M sodium chloride

0.05 M sodium acetate

4.5 mM zinc sulphate

20 ug / ml sonicated denatured salmon sperm DNA

pH adjusted to 4.6 using glacial acetic acid

The samples were heated to 95°C for 5 minutes before electrophoresis on a 6% denaturing polyacrylamide gel. The gel was run at a current of 22mA for a length of time dependent on

the size of probe and on the size of protected fragment expected. After electrophoresis the gel was autoradiographed overnight at -70°C.

2.13 S1 NUCLEASE ANALYSIS OF RADIOACTIVE RNA USING AN UNLABELLED DNA PROBE

In several experiments it was necessary to attempt to localise the 3' ends of radioactive precursor mRNAs, this was done using a modification of the standard S1 nuclease analysis. An unlabelled DNA probe was hybridised to the radioactive RNA and after S1 nuclease treatment the protected RNA fragment was analysed by polyacrylamide gel electrophoresis. This procedure was straightforward as long as the RNA to be analysed was labelled to a high specific activity and a large excess of the unlabelled DNA fragment was included. It was necessary to perform a series of test reactions to establish the optimum hybridisation temperature. Because the radioactivity detected in this procedure was in RNA rather than in DNA the sample in polyacrylamide gel loading buffer was only heated to 80°C prior to loading. The gel was electrophoresed and autoradiograped in the standard way.

2.14 ANALYSIS OF RADIOACTIVE PRECURSOR mRNAs BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Where radioactive precursor mRNAs were injected it was possible to analyse the results directly by electrophoresis of RNA samples. By comparing the size of the labelled RNA in the sample immediately after injection with the size of labelled RNA in the

sample taken after incubation of the injected oocytes it was possible to visualise directly whether cleavage of the precursor mRNA had occured. The success of this technique depended on the size of the precursor mRNA initially injected, this was because RNAs greater than about 1 kb ran on the acrylamide gels in the same position as the endogenous oocyte ribosomal RNAs, when this happened the labelled RNA appeared as a smear rather than as a discrete band, making analysis difficult. RNAs smaller than about 1 kb were very easy to analyse and large amounts of RNA, 1 - 2 oocytes worth, could be loaded onto a single gel track. For the larger RNAs 0.25 - 0.5 oocytes worth of RNA was the maximum that could be loaded per track to prevent smearing, consequently longer exposure times were necessary. Equivalent oocytes-worth of RNA from the samples to be compared were recovered by ethanol precipitation, vacuum dried and resuspended in 10 ul of polyacrylamide gel loading buffer, this amount of RNA was then loaded onto a "thick" 6% polyacrylamide gel without prior heating. After electrophoresis the gel was fixed in 10% glacial acetic acid and dried down before autoradiography. Where 1 - 2 oocytes worth of RNA was analysed a result was obtained in about 6 hours, for smaller amounts of RNA an exposure of longer than 48 hours was usually necessary.

2.15 OLIGO dT CELLULOSE BINDING ASSAY

Based on a method adapted from Maniatis et al, 1982, by R.W.Old. Oligo dT cellulose was used to isolate poladenylated RNAs from the bulk of the oocyte or embryo non - polyadenylated RNA. The oligo dT binding assay was used in 2 ways :

1. To fractionate RNA from oocytes injected with DNA into adenylated and non - adenylated fractions to determine if transcripts from the injected gene were adenylated or not.
2. To fractionate RNA from oocytes and embryos injected with labelled precursor mRNAs to investigate the adenylation status of the injected RNA.

For these experiments a large number of samples were fractionated therefore a batch binding procedure was used, this was much quicker and more convenient than column binding procedures. The RNA to be fractionated was dissolved in 10 - 20 ul of water. About 5 oocyte equivalents of RNA were used in each batch binding assay, it was more convenient to dissolve this amount of RNA in water rather than in oligo - dT loading buffer. The oligo - dT was equilibrated in oligo - dT loading buffer.

Loading buffer	Elution buffer
20 mM Tris / Cl pH 7.6	10 mM Tris / Cl pH 7.6
0.5 M sodium chloride	
1 mM EDTA	1 mM EDTA
0.1 % SDS	0.05% SDS

Sufficient oligo - dT cellulose to occupy a volume of about 300 ul was dispensed into a 1.5 ml eppendorf tube. The tube was centrifuged for 30 seconds to loosely pellet the oligo - dT. The loading buffer was removed to be replaced by 400 ul of fresh loading buffer. The RNA to be fractionated was then added and after mixing the tube was left at room temperature for 15 minutes with intermittent shaking. As a control for efficient binding radioactive RNA previously polyadenylated *in vitro* was used. This was bound in a parallel experiment and confirmed that the oligo - dT assay worked correctly. After binding of the RNA the tube was centrifuged for 30 seconds to pellet the oligo - dT cellulose and the bound RNA. The loading buffer containing unbound RNA was removed and stored until later. The oligo - dT cellulose was then washed 5 x with loading buffer to remove any non - adenylated RNA still remaining, it was centrifuged and the old loading buffer discarded after each wash. The polyadenylated RNA was then obtained by washing the oligo - dT cellulose with 2 x 500 ul aliquots of elution buffer, pre - warmed to 55°C. After the elution buffer was added the oligo - dt was thoroughly shaken and incubated at room temperature for about 5 minutes. The Tubes were then centrifuged and the bound fraction collected. Virtually all of the polyadenylated RNA came off in the first 2 fractions.

This method generated a 400 ul non - adenylated fraction and a 1 ml adenylated fraction containing equal oocyte equivalents of RNA. These fractions were briefly centrifuged to remove any oligo - dT and 10 ug of E.coli tRNA carrier was added to the adenylated fractions. RNA from each fraction was then recovered by ethanol

precipitation and resuspended for analysis. In experiments where the adenylation status of mRNA transcribed from injected genes was investigated both fractions were resuspended in S1 hybridisation buffer at a concentration of 5ul per oocyte equivalent of fractionated RNA. For experiments where the adenylation status of injected labelled RNAs were under investigation each fraction was resuspended in the same volume of polyacrylamide gel loading buffer for electrophoresis. The volume chosen depended on the specific radioactivity of the injected RNA.

2.16 PRIMER EXTENSION ANALYSIS

This technique was used to map the 5' ends of endogenous oocyte transcripts. A 5' end - labelled single stranded DNA primer complementary to a region of the transcript to be mapped was prepared. The gene coding for the transcript to be mapped was linearised within the coding region. A restriction enzyme was choosen that produced 5' overhangs. The 5' overhanging ends were labelled using the T4 kinase method. The labelled DNA was then restricted again with an enzyme that cut in the coding region of the gene, closer to the 5' end of the gene than the original enzyme and which generated 3' overhanging ends. The DNA fragments generated were electrophoresed on a denaturing acrylamide gel . The labelled strand of the primer fragment was separated from its unlabelled complementary strand on the basis of the size difference generated by the choice of restiction enzymes. The single stranded 5' end - labelled primer was eluted from an acrylamide gel slice and recovered by ethanol precipitation. For the mapping of endogenous oocyte transcripts 10 ug of RNA (2 oocyte equivalents) was used, this was resuspended in 5 ul of sterile water. The primer, also in sterile water. and 2 ul of 5 x hybridisation buffer were added to make a final volume of 10 ul. This mix was run into a drawn glass capillary.

5 x hybridisation buffer

2 M sodium chloride

50 mM PIPES pH 6.4

The capillary was incubated at 70°C for 3 hours to allow hybridisation to occur. The choice of the hybridisation temperature depended on the primer sequence and was determined by experiment. After hybridisation the mix was quickly cooled using iced water then expelled into 30 ul of ice cold primer extension mix.

Primer extension mix

0.5 mM dGTP, dCTP, dTTP and dATP
10 mM dithiothreitol
25 ug / ml actinomycin D
100 mM Tris / Cl pH 8
12 mM magnesium chloride

10 units of AMV reverse transcriptase were added and after mixing the reaction was incubated at 42°C for 1 hour. At the end of the reaction the template RNA was removed by the addition of 10 ul of 1 M sodium hydroxide and heating at 100°C for 5 minutes. 10 ul of 1 M HCl was then added to neutralise the alkali and after phenol extraction the extended primer was recovered by ethanol precipitation. The result was a mixture both of primer molecules that had not hybridised to the transcript and molecules that had hybridised and had been extended to the 5' end of the transcript. To analyse the extension products they were resuspended in 3 ul of polyacrylamide gel loading buffer and heated to 95 °C for 5 minutes before electrophoresis on a 6% denaturing acrylamide gel. After electrophoresis the gel was autoradiographed at - 70°C.

2.17 NORTHERN BLOTS

Based on Thomas, 1980.

a.) Formaldehyde agarose gel

This was poured on a standard size flat - bed gel plate set up in a fume hood. A 150 ml volume of 1.5% gel mix was made using 2.25 g of agarose, 112.5 ml of water and 15 ml of 10 x MOPS buffer. The mix was boiled to dissolve the agarose and allowed to cool to about 60°C. 22.5 ml of formaldehyde was then added and after mixing the gel was poured. Before use the formaldehyde was filtered through Whatman number 1 filter paper.

10 x MOPS buffer

0.2 M 3 - (N - morpholino) propanesulfonic acid (MOPS)

0.05 M anhydrous sodium acetate

0.01 M EDTA

Adjusted to pH 7 with sodium hydroxide.

The gel was run in a standard flat bed gel tank under 1 x MOPS buffer. The RNA samples were resuspended in water to give 2 oocyte equivalents (10 ug) RNA in 5 ul of water. The RNA was denatured prior to loading. The denaturing solution was made up by mixing 500 ul of deionised formamide with 100 ul of 10 x MOPS buffer and 150 ul of filtered formaldehyde. 15 ul of denaturing solution was added to the 5 ul of RNA. This mix was heated at 60°C for 5 minutes then quick cooled in iced water. 2 ul of RNA gel loading mix was added and the sample loaded onto the gel. The gel was electrophoresed at 100 V for about 5 hours.

RNA gel loading mix

50% glycerol

0.2% bromophenol blue

After electrophoresis the gel was soaked in 250 ml of 10% glycine for 20 minutes. 100 ul of 10 mg / ml ethidium bromide was added and the gel stained for 10 minutes. The gel was then rinsed in water for 2 x 15 minutes. The gel was photographed under U.V. light, to show the positions of the ribosomal RNA bands, before it was blotted.

The gel was soaked in 20 x SSC buffer for 20 minutes, the nitrocellulose for the blot was also soaked, first in water and then in 20 x SSC. The blot was then set up. The glass gel plate was supported on top of 2 plastic petrie dish lids in a small tray. The plate was covered with 4 thicknesses of 3 MM filter paper soaked in 20 x SSC, these overlapped the edges of the glass plate and were bent over to touch the bottom of the tray. 20 x SSC was put into the tray to fill it to a depth about ¼ way up the petrie dish supports. The gel was placed on top of the 3 MM paper and the nitrocellulose was carefully positioned on top of the gel. 2 thicknesses of 3 MM paper were put over the nitrocellulose followed by a large number of paper tissues to act as a wick. These tissues were covered with a second glass plate and weighed down. The areas between the edges of the gel and the edges of the tray were covered with "cling" film to prevent evaporation of the buffer. The gel was left overnight to blot. After blotting the nitrocellulose filter was baked for 2 hours at 80°C under vacuum and then stored at room temperature prior to probing.

Prehybridisation of the filter was carried out in a sealed polythene bag. The filter was placed in the bag along with about 15 ml of pre - hybridisation buffer. The bag was sealed and incubated at 42°C overnight.

pre - hybridisation buffer	1 x Denhardts
50% deionised formamide	0.02% w/v BSA
5 x SSC	0.02% polyvinylpyrolidone
50 mM sodium phosphate buffer pH 6.5	0.02% ficoll
200 ug / ml sonicated salmon sperm DNA	
1 x Denhardt's solution	Made as 50 x stock and stored frozen at - 20°C

The pre - hybridisation buffer was removed and replaced with an identical solution containing a labelled denatured DNA probe. The probe was prepared by nick - translation and then resuspended in sterile water. The probe was heated at 100°C for 5 minutes then quickly cooled using iced water. The denatured probe was added to cold hybridisation solution. 8 ml of hybridisation solution plus probe was added to each filter. The bag was resealed and the filter hybridised overnight at 42°C, this was done in a water bath with the bag held flat using a glass plate. After hybridisation the filter was removed from the bag and washed 4 times each time with 250 ml 2x SSC, 0.1 % SDS at room temperature. The filter was then washed twice with 250 ml of 0.1 x SSC, 0.1% SDS for 15 minutes per wash at 50°C. After washing the filter was covered with "cling" film and autoradiographed at - 70°C.

2.18 ADDITION OF POLY A TO PRECURSOR mRNA *IN VITRO*

Based on suppliers instructions (B.R.L.).

This method added poly A tails of heterogeneous lengths to the precursor mRNAs, after poly A addition these RNAs ran as smears on acrylamide gel electrophoresis rather than as discrete bands.

The SP6 RNA to be adenylated was made by the standard method, after the final ammonium acetate precipitation it was resuspended in 96 ul of sterile water. The method was suitable for any amount of RNA up to about 2 ug. The RNA in water was then added to the following reaction :

RNA	96 ul
2 x poly A mix	100 ul
100 mM Manganese chloride	2 ul
poly A polymerase	2 ul (about 7 units of enzyme)

2 x poly A mix

100 mM Tris / Cl pH 8

200 uM rATP

20 mM magnesium chloride

100 ug / ml BSA

0.5 M sodium chloride

The reaction was incubated at 37°C for 2 hours then stopped by the addition of EDTA to a final concentration of 20 mM. These reaction times could be varied along with the concentration of poly A polymerase to control the average poly A tail length. The polyadenylated RNA was phenol extracted, chloroform extracted and recovered by ethanol precipitation.

2.19 MOUSE GLOBIN mRNA PREPARATION

RNA rich in mouse globin mRNA was prepared from mouse red blood cells and used as a control for the S1 nuclease analysis of RNA from pAW103 injected oocytes. Blood was obtained from 2 mice by heart puncture and stored in cold PBS containing heparin at a concentration of 3 mg / ml.

PBS

140 mM sodium chloride

2.7 mM potassium chloride

8.1 mM Na_2HPO_4

1.5 mM KH_2PO_4

pH 7.4

The blood was centrifuged for 4 minutes in a MSE bench centrifuge at medium speed to pellet the red blood cells. The supernatant was removed leaving about 0.5 ml volume of cells. The cells were washed twice with cold PBS, between washes the cells were repelleted by gentle centrifugation. After washing the cells were lysed by the addition of 1 ml of water . The lysate was divided between 5 x 1.5 ml eppendorf tubes. 400 ul of Kressmans buffer containing proteinase K and SDS to 2% final was added to each tube. The tubes were incubated for 30 minutes at room temperature. The RNA preparation was then phenol extracted 2x, chloroform extracted 1 x and ether extracted 1 x before being recovered by ethanol precipitation. The amount of RNA obtained from 2 mice was resuspended in 500 ul of S1 hybridisation buffer, 10 ul of which was used per S1 nuclease analysis.

2.20 MODIFIED BARTHS' SALINE

This was a balanced salt solution used to store oocytes, eggs and embryos of Xenopus laevis. Usually 1 litre of solution was prepared using dechlorinated water. Where embryos were to be kept past gastrulation it was necessary to transfer them to a solution of 1/10 th strength prior to gastrulation. Modified Barths saline (M.B.S.) was routinely made up from 25 x stock solutions of M.B.S. A and M.B.S. B stored at - 20°C. After the M.B.S. was prepared the pH was tested, this was routinely around 7.8. Benzyl penicillin and streptomycin sulphate both at a concentration of 10 mg / litre were added to the M.B.S. solution when oocytes or embryos were to be incubated for long periods (more than 24 hours).

1 x M.B.S.

88.0 mM NaCl
1.0 mM KCl — M.B.S. A
2.4 mM $NaHCO_3$
15.0 mM HEPES - NaOH pH 7.4

0.3 mM $Ca(NO_3)_2.4H_2O$
0.41 mM $CaCl_2.6H_2O$ — M.B.S. B
0.82 mM $MgSO_4.7H_2O$

2.21 SURGICAL REMOVAL OF OOCYTES

This procedure was carried out by a holder of a home office animal experiment licence. Oocytes for injection were routinely obtained by surgically removing a small amount of Xenopus laevis ovary. The frog was anaesthetised using MS222 (0.15%), it was then laid on its back on a bed of ice. A small incision was made in the abdomen through both the outer skin and the abdomen wall at the position of the ovary. 2 - 3 lobes of ovary were removed by extracting them through the incision. The incision was repaired by 2 - 3 stitches in both the inner abdomen wall and the skin. The frog was transfered to recovery buffer (0.5 % sodium chloride) and left overnight to recover. The oocytes were quickly split into small clumps of 10 - 20, this was essential for their long term survival. Individual oocytes were separated manually from these small clumps and stored overnight prior to injection, this allowed oocytes damaged in the isolation procedure to be identified and discarded.

2.22 MATURATION OF OOCYTES *IN VITRO*

Where matured oocytes were required stage 6 oocytes were obtained and separated as described, they were then immediately incubated for about 16 hours in a solution of M.B.S. containing 20 ug / ml of progesterone. The progesterone was made up as a 2 mg / ml stock in ethanol and added to the M.B.S.. Matured oocytes were selected on the basis of a white spot on the animal hemisphere. Usually about 70% of the selected oocytes matured, those which did not were probably not genuine stage 6 oocytes. The matured oocytes

were transfered to a solution of M.B.S. without progesterone prior to injection.

2.23 *IN VITRO* FERTILISATION OF XENOPUS EGGS

Unfertilised eggs laid by hormonally induced females and these were fertilised *in vitro*. To induce the females to lay the following hormones were injected :

50 - 100 units of serum gonadotrophin B (FSH) 3 - 7 days prior to laying

500 - 600 units of human chorionic gonadotrophin 1 day prior to laying

The eggs were laid into dechlorinated water and collected for fertilisation. A male Xenopus was killed by injection of 0.5 ml of euthatal (sodium barbitone), the testis was removed and stored in a small amount of M.B.S. on ice. The testis was teased apart to release sperm before use. The eggs to be fertilised were transfered to a small dish using the wide end of a 10 ml pipette. Most of the liquid was removed and the testis was rubbed over the eggs for about one minute. 1 ml of water was then added to the dish to activate the sperm. The eggs were left about 1 minute for fertilisation to occur and then the dish was flooded with water. After about 15 minutes the fertilised eggs rotated with the black animal pole upwards. After rotation the jelly was removed from the fertilised eggs, this was done by swirling the eggs gently in a solution of 1% cysteine HCl (adjusted to pH 8 with NaOH) for about 15 minutes. Once the jelly was removed the embryos were

thoroughly rinsed and stored in M.B.S. ready for injection. The embryos were injected as soon as the first cleavage furrow was detected. Unfertilised eggs were sometimes required for injection, these were obtained from hormonally induced females and the jelly was removed using cysteine before they were injected.

2.24 MICROINJECTION TECHNIQUES

For microinjection of oocytes 5 - 10 oocytes were transfered to a glass microscope slide, each oocyte in its own drop of liquid. The oocytes were injected using a glass needle controlled by a paraffin oil filled syringe, operated by a micrometer. Injection was carried out under a binocular microscope. For nuclear injections the oocytes were positioned at an angle on the microscope slide with the animal pole facing the microinjection needle. The needle was then inserted into the centre of the animal pole just below the surface. A drop of liquid about 20 nl in volume was injected into the nucleus. The needle was then removed and the next oocyte injected. For cytoplasmic injections the needle was inserted into the vegetal pole well away from the nucleus. After injection the oocytes were kept in M.B.S. on ice for about 10 minutes which improved their survival, they were then incubated at 20 °C for the duration of the experiment.

Matured oocytes were injected in a similar way but here there was no germinal vesicle to aim for. For some experiments matured oocytes were injected in the animal pole and for others they were injected in the vegetal pole.

Embryos and unfertilised eggs were slightly more difficult to inject. Generally the techniques used were the same as for oocytes. For most experiments embryo injections were into 1 cell of an early 2 cell embryo. After injection the embryos were put immediately into M.B.S. at 20°C.

Microinjection needles were made by pulling hard glass capillaries over a micro - bunsen burner and then pulling them further into a fine point using a needle puller. Before use in RNA injection experiments the needles were heat treated at 225°C for 2 hours.

0.5 ul of the DNA or RNA sample, for injection, was spotted onto a square of "Nescofilm" and used to fill the injection needle. The remaining sample was kept on ice and used to refill the needle.

DNA injection experiments

DNA injections into the oocyte nucleus were performed in order to investigate the 3' processing of mRNA made *in vivo*. In these experiments circular plasmid DNA at a concentration of 500 ug / ml in water was used. About 20 nl of DNA solution was injected into each oocyte nucleus. After injection the oocytes were incubated at 20°C for approximately 20 hours for transcription to occur. In each individual injection experiment about 20 - 30 oocytes were injected. After incubation the injected oocytes were homogenised and RNA prepared for analysis.

RNA injection experiments

Many experiments were carried out where radioactively labelled precursor mRNAs were injected into oocytes, matured oocytes, eggs

and embryos. The principle behind all these experiments was very similar. A batch of oocytes or embryos were injected with the precursor mRNA. A sample of injected oocytes or embryos was homogenised immediately after injection and other samples were taken at further times after injection. By comparing the later samples with the initial sample changes in size of the precursor mRNA were followed. In practice the first time point sample was taken after some of the oocytes in the batch had been injected for about 20 minutes whereas others had only just been injected. It was therefore sometimes possible to detect a small amount of processed RNA in the first time point sample. α ^{32}P - SP6 RNA was resuspended in water at a concentration of 100 ug / ml, approximately 20 nl of this solution was injected into each oocyte, egg or embryo.

Oocyte nuclear and cytoplasmic injections were performed as described for the DNA injection experiments. RNA samples from the injected oocytes were prepared from immediately after injection and from 3 or 5 hours after injection.

Injections into matured oocytes and unfertilised eggs were also carried out using the same procedure and RNA samples were prepared from the same times after injection. Matured oocytes and eggs were activated by injection and began a series of abortive cleavages. The majority of injected matured oocytes remained healthy, as judged by their external appearance, for the course of these experiments. Only healthy injected matured oocytes and eggs were selected to be homogenised.

Embryos were injected into 1 cell of the 2 cell embryo. Samples were taken from immediately after injection and from 7 hours after injection.

2.25 PREPARATION OF RNA SAMPLES FROM OOCYTES, EGGS AND EMBRYOS

This method was based on Probst et al, 1979.

Samples usually of 10 oocytes or embryos were taken in the same way in each DNA and RNA injection experiment, these samples were used to prepare total RNA for analysis. The 10 oocytes to be homogenised were transfered to a 1.5 ml eppendorf tube. Any M.B.S. was removed and 500 ul of Kressmann's buffer containing 2% SDS was added. Homogenisation was achieved by pipetting the oocytes up and down a blue "Gilson" tip several times. The sample was then incubated at room temperature for 45 minutes.

Kressmann's buffer

10.0 mM Tris / Cl pH 7.4

1.5 mM magnesium chloride

10.0 mM sodium chloride

1 mg / ml proteinase K

After treatment with Kressmann's buffer the sample was phenol extracted twice, chloroform extracted twice and ether extracted. The RNA was then recovered by ethanol precipitation. For experiments where DNA had been injected and RNA was to be analysed by S1 nuclease analysis the precipitated RNA was resuspended at 1 mg / ml in S1 hybridisation buffer. For experiments where radioactive precursor mRNAs had been injected amounts of RNA from equal numbers of injected oocytes were always compared.

Sub - clones in pSP64 and pSP65

Sub - clones, from several genes, were made in SP6 plasmids in order that they could be used to make precursor mRNAs *in vitro*. Details of these sub - clones are shown below :

2.26 SUB - CLONES OF THE MOUSE β (MAJOR) GLOBIN GENE

2 sub - clones of regions of this gene were made in the plasmid pSP64 (see figure 1). The source of the globin sequences was a hybrid gene, pAW103, consisting of the promoter and 5' end of a *Xenopus* histone H3 gene joined to the 3' end of the mouse β globin gene (Wilson, 1986). A map of pAW103 is shown in figure 2A with the positions of various restiction enzyme sites marked. Figure 2.B shows a portion of the globin sequence in pAW103 including the globin mRNA 3' end position. The 2 sub - clones containing globin sequences were called pSP64.MG1 and pSP64.MG2. Details of pSP64.MG1 are shown in figure 3. To construct pSP64.MG1 a 392 nt Sau 3A fragment, spanning the globin mRNA 3' end position, was isolated from pAW103. This Sau 3A fragment was then cloned into the Bam HI site of the pSP64 polylinker. The insert in pSP64.MG1 contained 246 nt of sequence upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of the mRNA 3' end position. The orientation of the insert relative to the SP6 promoter was confirmed using an asymmetric Rsa I site in the globin sequence as the basis for a diagnostic digest (see figure 4). The pSP64 plasmid contained 3 Rsa I sites and when digested with this enzyme gave rise to 3 DNA fragments. The Sau 3A globin fragment cloned in pSP64.MG1 contained a single Rsa I site, this site was 106 nt downstream

from the Sau 3A site in the coding region of the globin gene (figure 4a). The Rsa I site in pSP64 closest to the SP6 promoter was 112 nt upstream from the Bam HI site in the SP6 polylinker (figure 4b). If the globin insert was in the required orientation a Rsa I digest of the pSP64.HG1 sub - clone was expected to generate an extra Rsa I fragment over the pSP64 Rsa I digest. The extra fragment would have been 218 nt in length (figure 4c). If the globin insert was in the opposite, unwanted, orientation a Rsa I digest of the sub - clone in pSP64 would have generated an additional DNA fragment 396 nt in length (figure 4d). The results of Rsa I digests of pSP64.HG1 and pSP64 alone are shown in figure 4e. The pSP64.HG1 sub - clone was shown to generate an extra fragment approximately 220 nt in length when digested with Rsa I. The size of this fragment was obtained by comparison with the migration of DNA markers of known sizes. The extra Rsa I fragment was clearly not 396 nt in length. It was concluded that the Sau 3A globin fragment cloned in pSP64.HG1 was in the orientation shown in figure 4c and would generate mRNA - like transcripts on transcription. A number of other restriction digests were also performed which supported this conclusion (data not shown). In the pSP64.HG1 Rsa I digest ,shown in figure 4e, the remainder of the globin sequence from the Rsa I site in the insert to the next Rsa I site in pSP64 downstream of the polylinker was added onto the largest vector fragment in the pSP64 digest. Hence this large fragment migrated more slowly in the Rsa I digest of pSP64.HG1. Figure 4e also shows the results of a Hind III / Ava I double digest of pSP64.HG1, both of these

enzymes did not cut in the Sau 3A globin fragment. Hind III cut in the SP6 polylinker upstream of the insert and Ava I cut in the SP6 polylinker downstream of the insert. The double digest in effect removed the globin sequence cloned in the Bam HI site of the polylinker. The size of this fragment was about 419 nt as expected. Further confirmation that the pSP64.MG1 clone did contain the required globin fragment was obtained from the fact that it could be used to make a 3' end - labelled probe for S1 nuclease analysis. A Sau 3A 3' end - labelled probe, made from the pSP64.MG1 sub - clone, generated a protected fragment of the expected size when hybridised to authentic mouse globin transcripts (see section 3.5).

Details of the pSP64.MG2 sub - clone are given in figure 5. A 1.3 kb Hind III / Xba I fragment from pAV103 was cloned into the Hind III and Xba I sites of pSP64. In theory this fragment was very likely to be cloned in the required orientation. Figure 6a shows the Hind III and Xba I sites in pAV103, figure 6b shows the globin fragment cloned in pSP64.MG2. The orientation of this clone was confirmed by the restriction enzyme digests shown in figure 6 c. A Pst I site located 387 nt downstream from the Hind III site in the globin sequence was used. Pst I and other restriction enzymes that cut in the SP6 polylinker either upstream or downstream of the insert were used in double restriction digests. Hind III cut at the first restriction site in the SP6 polylinker downstream from the SP6 promoter. A Hind III / Pst I double digest generated a 387 nt fragment from the globin gene and a long fragment consisting of pSP64 vector

sequence plus the remaining 920 nt from the globin insert. Xba I and Eco RI both cut in the SP6 polylinker downstream of the globin insert. Pst I / Xba I and Pst I / Eco RI double digests both generated fragments of similar size. Both digests produced a fragment of approximately 920 nt and a long vector fragment. The 920 nt fragment in each case was the size of the distance between the Pst I site in the globin insert and either the Xba I site or the Eco RI site downstream of the insert. The vector fragment from these digests contained the SP6 vector sequence plus the remainder of the globin sequence not in the Pst I / Xba I or Pst I / Eco RI fragments. These digests confirmed that the globin Hind III / Xba I fragment was cloned in pSP64 in the correct orientation to produce mRNA - like transcripts. Figure 6 also shows the results of Hind III / Xba I double digests of both pSP64.MG2 and pAW103, these digests demonstrated that the insert in pSP64.MG2 did correspond in size to the required Hind III / Xba I fragment from pAW103.

2.27 SUB - CLONES OF THE XENOPUS hsp70A GENE IN pSP65

A sub - clone of a region from the Xenopus hsp 70A gene (Bienz, 1984), spanning the mRNA 3' end position, was made in pSP65. This hsp 70A sub - clone was called pSP65.HS1. The hsp 70A gene had been cloned in the plasmid pUC 12. A 1.16 kb Sst I / Pst I fragment containing the required region was isolated from the pUC 12 hsp 70A plasmid (see figures 7 and 8). The Sst I / Pst I fragment was then cloned downstream of the SP6 promoter in the Sst I and Pst I sites of the pSP65 polylinker (figure 1.B). The

cloned fragment contained about 600 nt of sequence upstream of the hsp 70A mRNA 3' end position and about 560 nt of sequence from downstream of this position. As a consequence of the cloning strategy used it was very likely that the fragment would have been cloned in the required orientation, that is with the Sst I end closest to the SP6 promoter. Restriction digests, shown in figure 9, were performed to confirm the orientation of the insert in pSP65.HS1. The heat shock fragment contained a single Pvu II site. This Pvu II site was located 270 nt downstream of the Sst I site and approximately 890 nt upstream from the Pst I site (figure 9a). The Pvu II site was used in combination with a unique Pvu II site in the pSP65 vector sequence located downstream of the polylinker. If the hsp 70A fragment was cloned in the required orientation a Pvu II digest was expected to generate 2 fragments. One fragment would have been approximately 970 nt in length, the size being the distance between the Pvu II site in the heat shock insert to the Pvu II site downstream of the SP6 polylinker. The second larger fragment would have consisted of the SP6 vector plus part of the heat shock insert. If the hsp 70A insert had been cloned in the opposite orientation the smallest insert fragment would have been only about 350 nt in length because the Pvu II site in the heat shock insert would have been closer to the Pvu II site downstream of the SP6 polylinker. The second fragment would have comprised of the vector sequence plus the remainder of the heat shock insert. The result of a Pvu II digest of pSP65.HS1 is shown in figure 9b. This digest produced 2 fragments, one of which was approximately

970 nt long and the other of which was greater than 3 kb long. This result confirmed that the insert in pSP65.HS1 was in the orientation required to produce mRNA - like transcripts. Further restriction enzyme digests were also performed on pSP65.HS1. A Sst I / Pst I double digest cut out the insert from the SP6 polylinker. Other restriction enzymes which cut only once in the pSP65 vector and did not cut in the cloned heat shock sequence each linearised the pSP65.HS1 sub - clone as was expected, these enzymes included Sst I, Pst I and Hind III.

Fig. 1

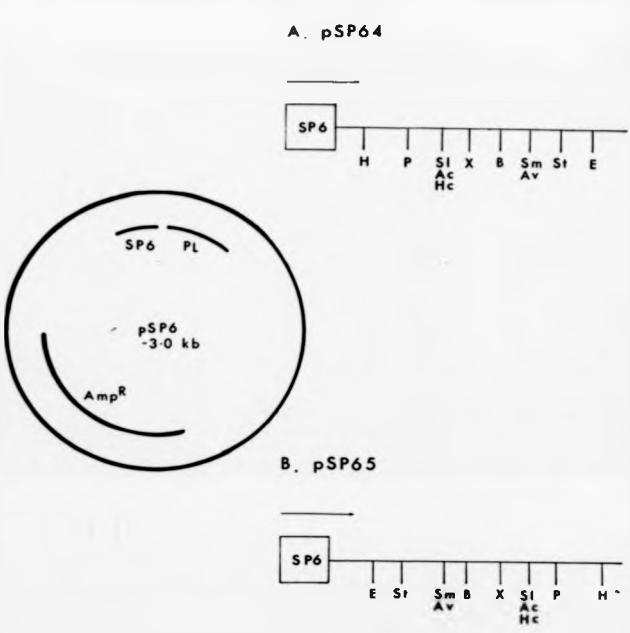

A. pSP64
SP6
H
P
Sl
Ac
Hc
X
B
Sm
Av
St
E
SP6
PL
pSP6
-3·0 kb
Amp^R
B. pSP65
SP6
E
St
Sm
Av
B
X
Sl
Ac
Hc
P
H

Figure 1. SP6 vectors pSP64 and pSP65 (Melton et al, 1984).

These vectors are about 3.0 kb in length and contain a polylinker sequence (PL) cloned downstream of the SP6 promoter (SP6). DNA fragments cloned into the polylinker sites can be transcribed *in vitro* using SP6 polymerase. Both vectors also contain the ampicillin resistance gene (amp^r) as a selectable marker. The vectors differ in the orientation of the polylinker sequence with respect to the SP6 promoter. Figure 1.A shows the order of restriction enzyme sites in pSP64 and figure 1.B shows the order of restriction enzyme sites in pSP65.

Key to restriction enzyme abbreviations :

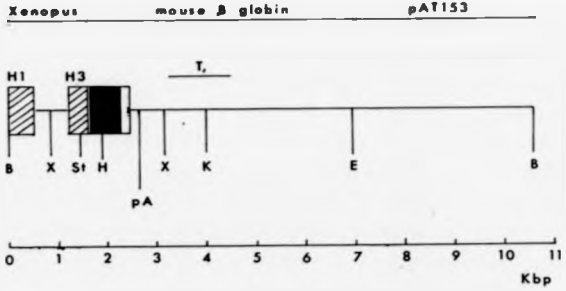
H = Hind III P = Pst I Sl = Sal I Ac = Acc I

Hc = Hinc II X = Xba I B = Bam HI Sm = Sma I

Av = Ava I St = Sst I E = Eco RI

Fig. 2

A. pAW103



B. The 3' end of the mouse β globin gene

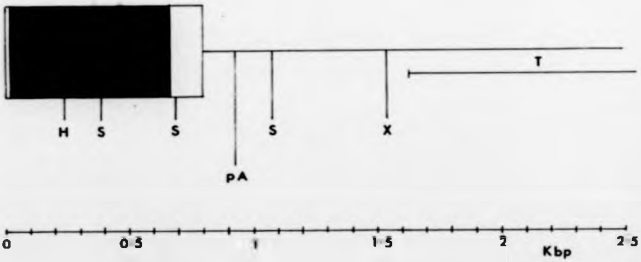


Figure 2. Restriction enzyme map of pAW103

Figure 2.A. The entire pAW103 clone (Wilson, pers. comm.) with the Xenopus histone sequences, the mouse globin sequences and the pAT153 vector sequences marked.

Figure 2.B. A portion of the mouse globin gene, from pAW103, including the globin mRNA 3' end position.

The blacked - out portion of the globin gene represents an intron sequence.

T = Transcription termination region (Citron et al, 1984).

pA = Site of polyadenylated 3' terminus of mouse globin mRNA.

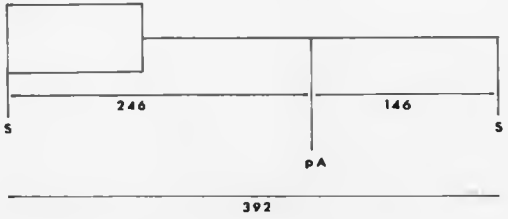
Key to restriction enzyme abbreviations :

B = Bam HI X = Xba I St = Sst I H = Hind III

K = Kpn I E = Eco RI S = Sau 3A

Fig. 3

A. 392 bp S fragment from pAW103



B. Cloned in pSP64

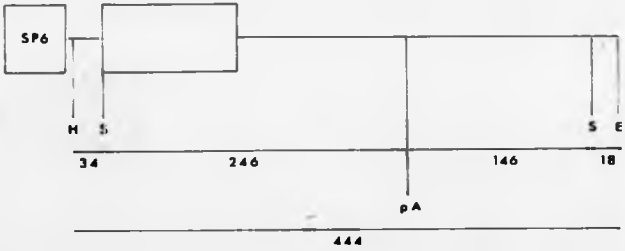


Figure 3. The mouse globin DNA fragment cloned in pSP64.MG1

Figure 3.A The 392 Sau 3A fragment spanning the mouse β globin mRNA 3' end position (pA). This fragment contained 246 nt of sequence from upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of this position.

Figure 3.B The 392 nt Sau 3A fragment cloned into the Bam HI site of pSP64. When linearised with Eco RI and transcribed with SP6 polymerase this sub - clone generated a 444 nt full length precursor mRNA. The precursor mRNA comprised of the globin sequence plus small amounts of SP6 polylinker sequence.

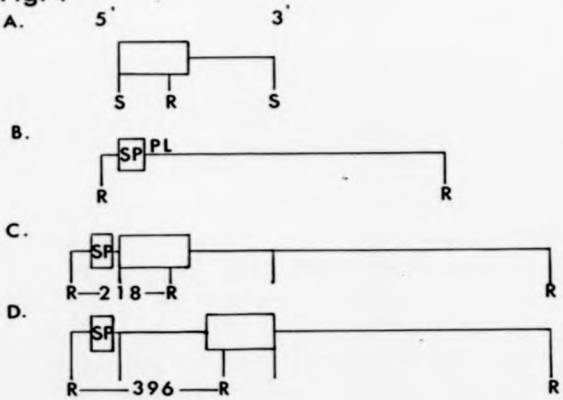
SP6 = SP6 promoter.

Key to restriction enzyme abbreviations :

H = Hind III S = Sau 3A E = Eco RI

All sizes marked are in nucleotides.

Fig. 4


A.
5'
3'
S
R
S
B.
SP
PL
R
R
C.
SP
R—218—R
R
D.
SP
R——396——R
R

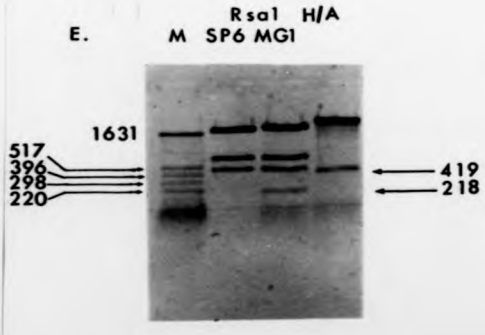

E.
RsaI H/A
M SP6 MG1
1631
517
396
298
220
419
218

Figure 4. The orientation of the globin insert in pSP64.MG1

Figure 4.A. The 392 nt Sau 3A fragment from pAW103.

Figure 4.B. The position of the Rsa I restriction enzyme sites upstream and downstream of the polylinker in the pSP64 vector.

Figure 4.C. The 392 nt Sau 3A fragment cloned in pSP64 in the required orientation, note this generates an extra Rsa I fragment 218 nt in length.

Figure 4.D. The 392 nt Sau 3A fragment cloned in pSP64 in the opposite, unwanted, orientation, note this generates an extra Rsa I fragment 396 nt in length.

Abbreviations of restriction enzyme sites :

S = Sau 3A R = Rsa I

Figure 4.E. An agarose gel showing test digests on pSP64.MG1 DNA to confirm the orientation is as shown in figure 4.C.

M = pAT153 marker digest.

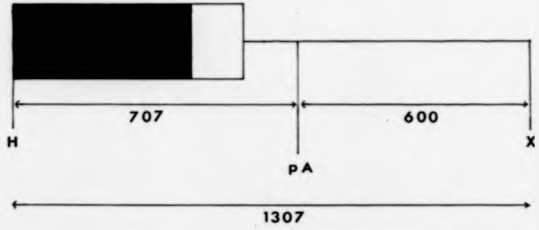
Rsa I / SP6 = Rsa I digest of pSP64 vector.

Rsa I / MG 1 = Rsa I digest of pSP64.MG1.

H / A = Hind III / Ava I double digest of pSP64.MG1

Fig. 5

A. 1307 bp H/X fragment from pAW103



B. Cloned in pSP64

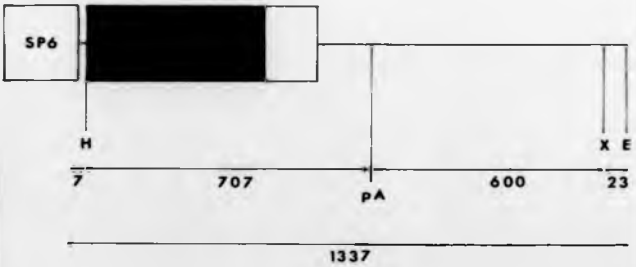


Figure 5. The globin DNA fragment cloned in pSP64.MG2

Figure 5.A. The 1307 nt Hind III / Xba I fragment from pAW103 spanning the globin mRNA 3' end position (pA).

Figure 5.B. The Hind III / Xba I globin fragment cloned in the Hind III and Xba I sites of pSP64.

The blacked out area of the globin gene represents an intron sequence.
SP6 = SP6 promoter.

Key to abbreviations of restriction enzyme sites :

H = Hind III X = Xba I E = Eco RI

Fig. 6

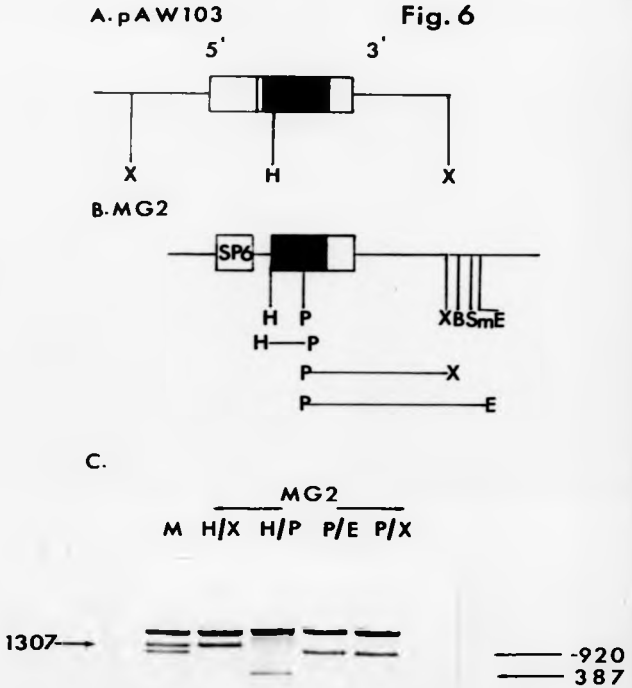

A. pAW103
5'
3'
X
H
X
B. MG2
SP6
H P
XBSmE
H—P
P——X
P——E
C.
MG2
M H/X H/P P/E P/X
1307
920
387

Figure 6. The orientation of the globin insert in pSP64.MG2

Figure 6.A. pAW103 with Hind III and Xba I restriction enzyme sites labelled.

Figure 6.B. pSP64.MG2, the SP6 vector with the globin Hind III / Xba I fragment cloned into the Hind III and Xba I sites.

Key to abbreviations of restriction enzyme sites :

X = Xba I H = Hind III P = Pst I B = Bam HI
Sm = Smm I E = Eco RI

M = pAW103 Hind III / Xba I digest.
MG2 H/X = pSP64.MG2 Hind III / Xba I digest.
MG2 H/P = pSP64.MG2 Hind III / Pst I digest.
MG2 P/E = pSP64.MG2 Pst I / Eco RI digest.
MG2 P/X = pSP64.MG2 Pst I / Xba I digest.

Fig. 7

Xenopus heat shock gene hsp70A

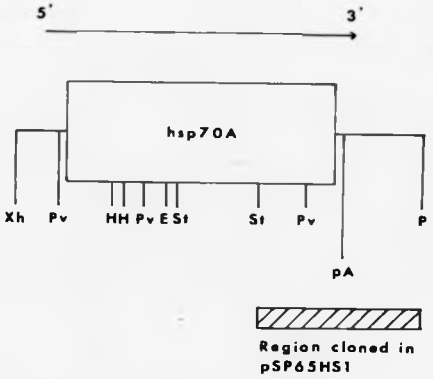


Figure 7. The Xenopus heat shock gene hsp70A (Bienz, 1984)

pA = mRNA 3' end position.

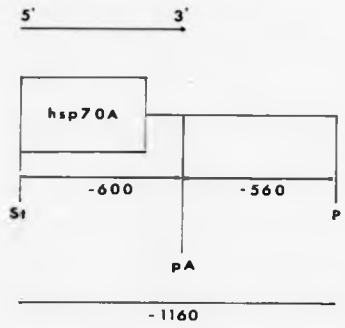
Key to abbreviations of restriction enzyme sites :

Xh = Xho I Pv = Pvu II H = Hind III E = Eco RI

st = Sst I P = Pst I

Fig. 8

A. The 3' end of the Xenopus hsp70A gene



B. Cloned in pSP65

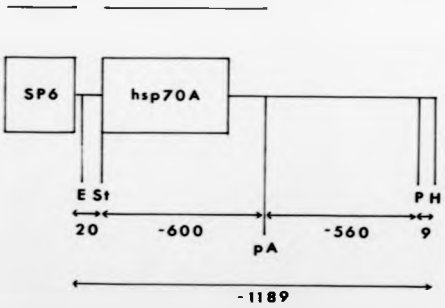


Figure 8. The hsp70A DNA fragment cloned in pSP65.HS1

Figure 8.A. The Sst I / Pst I restriction enzyme fragment spanning the mRNA 3' end position (pA) of the Xenopus hsp70A gene.

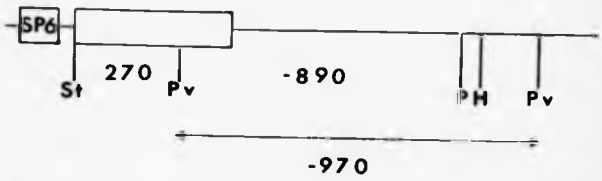
Figure 8.B. The 1160 nt Sst I / Pst I hsp70A fragment cloned in the Sst I and Pst I restriction sites of pSP65, this made the sub - clone pSP65.HS1.

Key to abbreviations of restriction enzyme sites :

St = Sst I P = Pst I E = Eco RI H = Hind III

All sizes shown are in nucleotides.

Fig. 9
A. pSP65.HS1



B.

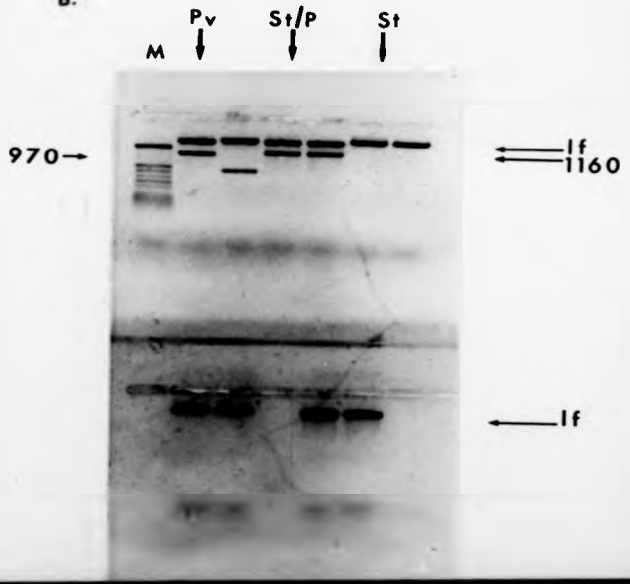


Figure 9. The orientation of the hsp70A DNA fragment in pSP65.HS1

Figure 9.A. The Sst I / Pst I fragment from the hsp70A gene cloned in pSP65.

Key to abbreviations of restriction enzyme sites :

St = Sst I Pv = Pvu II P = Pst I H = Hind III

Figure 9.B. An agarose gel showing restriction enzyme test digests of pSP65.HS1.

M = pAT153 Hinf I marker digest.
Pv = pSP65.HS1 Pvu II digest.
St/P = pSP65.HS1 Sst I / Pst I digest.
St = pSP65.HS1 Sst I digest.

Lower gel :
H = pSP65.HS1 Hind III digest.
P = pSP65.HS1 Pst I digest.
lf = linearised pSP65.HS1.

2.28 SUB - CLONES OF XENOPUS H4 AND H1 HISTONE GENES IN pSP64

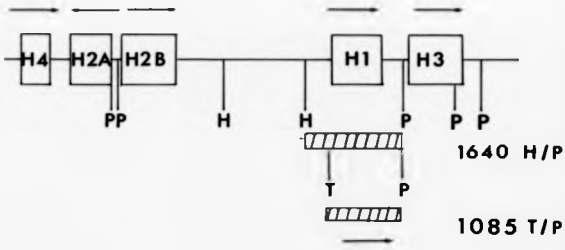
These sub - clones were made by D.Jackson as part of a Ph.D. project (Jackson, pers. comm.). 2 sub - clones , one containing a Xenopus H1 gene and one containing a Xenopus H4 gene were made in pSP64. These histone sub - clones were named pSP64.X1H1 and pSP64.X1H4 respectively. pSP64.X1H1 contained a 1085 nt Taq I / Pst I fragment from the histone gene cluster pX1HV8 (Old et al, 1982), see figure 10. This fragment was isolated and the ends of the fragment were end-filled. Hind III linkers were then added and a Hind III / Pst I double digest was performed. This had the affect of creating a Hind III end at the Taq I end of the fragment. The hind III / Pst I fragment containing the H1 gene was then inserted into the Hind III and Pst I sites in pSP64 (see figure 11), this generated a clone in the correct orientation to produce mRNA - like transcripts.

pSP64.X1H4 contained a 714 nt Sal I / Pst I H4 fragment from the histone gene cluster pX1HV23 (Old et al, 1982). The location of the Sal I / Pst I fragment in pX1HV23 is shown in figure 10. The ends of the Sal I / Pst I fragment were blunted and Hind III linkers were added. The Hind III linked fragment was digested with Hind III and Pst I and cloned in the Hind III and Pst I sites of the pSP64 polylinker (see figure 12), this generated a clone in the correct orientation to produce mRNA - like transcripts.

Both clones were extensively tested by restriction enzyme digests (Jackson, pers comm and other data not shown). In addition it

Fig.10

A. pXIHW8



B. pXIHW23

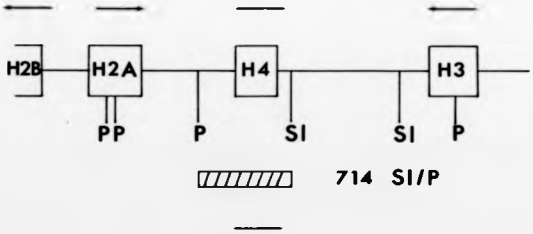


Figure 10. The Xenopus histone gene clusters pX1HW6 and pX1HW23 (Old et al. 1982).

Figure 10.A. The pX1HW6 histone gene cluster.

1640 H/P = 1640 nt Hind III / Pst I fragment containing the H1 histone gene.
1085 T/P = 1085 nt Taq I / Pst I fragment containing the H1 gene to be cloned in pSP64.X1H1.

Figure 10.B. The pX1HW23 histone gene cluster.

714 S1/P = 714 nt Sal I / Pst I fragment containing the H4 gene to be cloned in pSP64.X1H4.

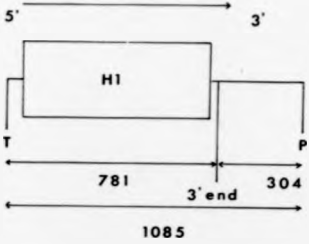
Key to abbreviations of restriction enzyme sites :

P = Pst I H = Hind III T = Taq I S1 = Sal I

Fig. 11

A.

1085 bp T/P H1 fragment from pX1HW8



B.

H linkers added, H/P digests, cloned in pSP64

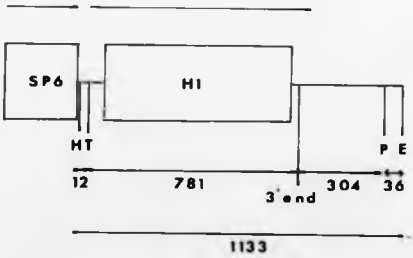


Figure 11. The Xenopus H1 histone fragment cloned in pSP64.X1H1

Figure 11.A. The 1085 nt Taq I / Pst I fragment containing the H1 gene isolated from pX1HW8.

Figure 11.B. The H1 fragment cloned in pSP64 to generate the sub - clone pSP64.X1H1.

SP6 = SP6 promoter 3' end = H1 mRNA 3' end position.

Key to abbreviations of restriction enzyme sites :

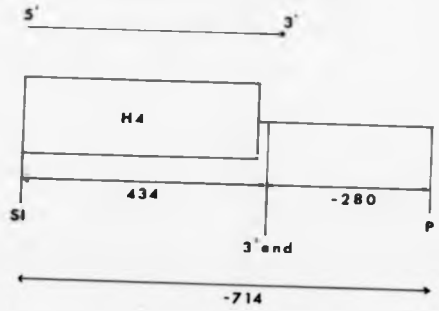
T = Taq I P = Pst I H = Hind III E = Eco RI.

All sizes shown are in nucleotides

Fig.12

A.

714 bp SI/P H4 fragment from pX1HW23



B. H linkers added, H/P digests, cloned in pSP64

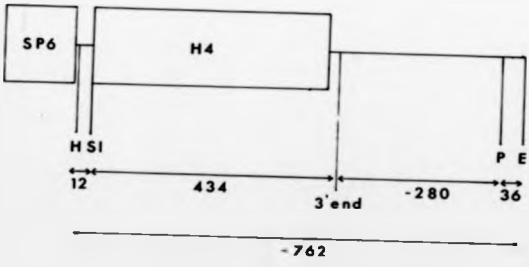


Figure 12. The Xenopus histone H4 fragment cloned in pSP64.X1H4

Figure 12.A. The 714 nt Sal I / Pst I fragment containing the H4 gene isolated from pX1HW23.

Figure 12.B. The H4 fragment cloned in pSP64 to generate the sub - clone pSP64.X1H4.

Key to abbreviations of restriction enzyme sites :

S1 = Sal I P = Pst I H = Hind III E = Eco RI

SP6 = SP6 promoter 3' end = H4 mRNA 3' end position.

All sizes shown are in nucleotides.

was shown that the H4 sub - clone could be used to make a radioactive primer which was extended as expected when hybridised to authentic oocyte H4 transcripts (see section 3.1).

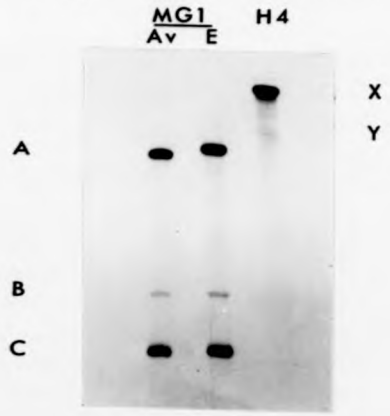
2.29 TRANSCRIPTION PRODUCTS OF SP6 SUB - CLONES

SP6 transcriptions of the histone and globin sub - clones, described above, were performed in the presence of α ^{32}P rGTP. A small amount of the transcription products from each sub - clone were then analysed by polyacrylamide gel electrophoresis followed by autoradiography. Figure 13 shows the transcription products from pSP64.X1H4 linearised with EcoRI, pSP64.MG1 linearised with Eco RI and pSP64.MG1 linearised with Ava I. pSP64.X1H4 produced one major transcript (labelled x) which was the result of full length transcription of the inserted H4 sequence, it also produced 2 very minor transcription products (labelled Y). pSP64.MG1 produced 2 major transcripts (labelled A and C) and a minor transcript (labelled B). A was the full length transcription product whereas B and C were presumably caused by premature transcription termination by the SP6 polymerase (see appendix). The sizes of B and C were calculated from a comparison of their migration wiith the migration of single stranded DNA markers of known sizes. B was calculated to be approximately 250 nt long and C was calculated to be about 215 nt long. Approximately 50% of the pSP64.MG1 transcripts were in the full length band and only these RNAs would have contained extensions downstream of the globin mRNA 3' end position. Both

transcripts B and C terminated before the globin mRNA 3' end position.

Figure 14 shows the transcription products from the pSP64.MG2 sub - clone. This sub - clone generated 3 major transcripts labelled a, b and c. a was the full length transcript and b and c were presumably caused by premature transcription termination by the SP6 polymerase (see appendix). The sizes of b and c were calculated graphically but this was not very accurate because of the lack of markers of known size in this region of the gel. The size of RNA b was more accurately estimated from figure 41 where pSP64.X1H4 and pSP64.MG2 RNAs were run on the same gel. RNA b was shown to be considerably longer than the full length pSP64.X1H4 transcript. RNA b was therefore about 800nt or longer in length. The distance from the 5' end of pSP64.MG2 transcripts to the globin mRNA3' end position was about 714 nt, therefore RNA b, as well as RNA a, contained an extension downstream of the globin mRNA 3' end position. RNA c terminated upstream of the globin mRNA 3' end position (see appendix). Greater than 50% of the transcription products from pSP64.MG2 were therefore potential substrates for an RNA 3' end processing reaction.

Fig. 13
pSP64.XIH4 and pSP64.MG1 RNAs



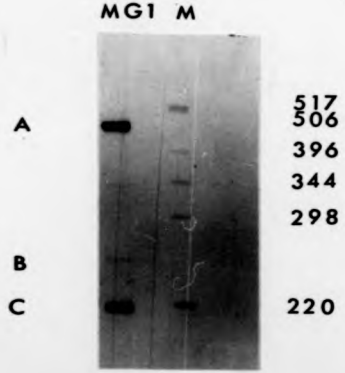


Figure 13. SP6 transcription products from pSP64.X1H4 and pSP64.MG1

Polyacrylamide gel electrophoresis of total transcription products from SP6 sub - clones.

MG1 Av = Transcription products from pSP64.MG1 linearised with Ava I.

MG1 E = Transcription products from pSP64.MG1 linearised with Eco RI.

H4 = Transcription products from pSP64.X1H4 linearised with Eco RI.

M = pBR322 Hinf I size marker.

A = Full length MG1 transcripts, B and C - shorter MG1 transcripts.

X = Full length H4 transcripts, Y = shorter H4 transcripts.

Fig. 14

pSP64.MG2 transcription products

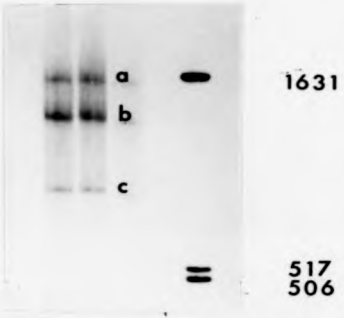


Figure 14. SP6 transcription products of the pSP64.MG2 sub - clone

pSP64.MG2 was linearised with Eco RI and transcribed with SP6 polymerase. The total transcription products were analysed by polyacrylamide gel electrophoresis.

RNA = Products of pSP64.MG2 transcription.
M = pBR322 Hinf I size marker.

a = Full length MG2 transcript, b and c = shorter MG2 transcripts.

SECTION 3

RESULTS AND DISCUSSION

3.1 3' CLEAVAGE AND POLYADENYLATION OF HISTONE PRECURSOR mRNAs IN THE XENOPUS OOCYTE

a. Introduction

The fully grown Xenopus oocyte (stage 6) has been widely used as an assay system to investigate histone mRNA 3' end formation, this work includes experiments in which artificial precursor RNAs were shown to undergo cleavage at the mRNA 3' end position after microinjection into the oocyte nucleus. This cleavage reaction has been demonstrated for precursors to the sea urchin H2A mRNA (Birchmier et al, 1984) and the chicken H2B mRNA (Krieg and Melton, 1984). Later work has shown that Xenopus H4 precursor mRNAs undergo 3' cleavage in the oocyte nucleus (Georgiev et al, 1984) and that sea urchin H3 precursor mRNAs undergo 3' cleavage in oocytes preinjected with the U7 snRNA (Georgiev and Birnstiel, 1985).

In this chapter experiments using Xenopus H4 and H1 genes to make artificial precursor mRNAs for oocyte injection experiments are described. These precursor mRNAs were tested for 3' cleavage in both the nucleus and cytoplasm of stage 6 oocytes. An investigation was also made into the polyadenylation states of precursor and 3' cleaved RNAs after injection into the oocyte. It has been reported that endogenous Xenopus oocyte H4 mRNAs possess short (0 - 20 A's) poly A tracts at their 3' ends (Turner and Woodland, 1982, Ballantine and Woodland, 1985), this was reinvestigated using the same oligo - dT cellulose binding assay

as was used to determine the polyadenylation status of the injected histone RNAs, this was a necessary control to confirm that the oligo dT assay was sensitive enough to distinguish RNAs with short A tracts from non - polyadenylated RNAs.

b. Results

The plasmids pSP64.X1H4 and pSP64.X1H1 were used to make artificial precursor RNAs. These plasmids are described in detail in the materials and methods section. pSP64.X1H4 contains a 714 nt fragment from a Xenopus H4 gene cloned downstream from the SP6 promoter, this fragment includes 434 nt of sequence from the H4 gene up to mRNA 3' end position and 280 nt of sequence from past this position (see figure 12). pSP64.X1H4 was linearised with Eco RI and transcribed using SP6 polymerase. A cap analogue and a labelled nucleotide were included in the mixture. The result was an artificial precursor mRNA that was radioactively labelled and had a cap structure at the 5' end. This RNA was 762 nt long due to the presence of SP6 polylinker sequences at the 5' and 3' ends. PSP64.X1H1 contains a 1085 nt fragment from a Xenopus H1 gene, this fragment includes 781 nt of sequence up to the mRNA 3' end position and 304 nt of sequence from past this position (see figure 11). When linearised and transcribed with SP6 polymerase this plasmid produced a precursor mRNA 1133 nt long.

These labelled precursor mRNAs were tested for 3' cleavage in the oocyte nucleus and cytoplasm. Each RNA was injected into the required compartment of about 20 oocytes. Immediately after injection half of the injected oocytes were homogenised and total

RNA, incuding the injected labelled RNA, was prepared. The remaining oocytes were incubated for 3 hours then homogenised and total RNA was prepared. These RNA samples were analysed directly by acrylamide gel electrophoresis. In these experiments, although total RNA was loaded onto the gels, only the injected precursor mRNA and it's derivatives were labelled so only these RNAs were detected by autoradiography of the gels. For each experiment the samples loaded onto the gels contained RNA from an equal number of injected oocytes so they could be compared. The results of this experiment are shown in figure 15.

Both the H4 and H1 precursor mRNAs underwent 3' cleavage in the oocyte nucleus to produce RNAs of a size consistent with that cleavage being at the mRNA 3' end position. For the H4 RNA this involved conversion of the 762 nt precursor mRNA to a 446 nt 3' cleaved RNA. For the H1 RNA the precursor mRNA was 1133 nt and the 3' cleaved RNA was 793 nt. In both cases approximately 50% of the injected precursor RNA was 3' cleaved. The result of injection of the H4 precursor mRNA into the oocyte cytoplasm is also shown in this figure. In the cytoplasm the RNA was relatively stable but did not undergo the 3' cleavage reaction.

The RNA samples obtained from this experiment were then used in an oligo - dT cellulose binding assay to determine whether the 3' cleaved RNAs were polyadenylated and whether the precursor mRNAs acted as substrates for polyadenylation activity. Each RNA sample was bound to oligo - dT cellulose using a batch binding method and adenylated and non - adenylated fractions were obtained. Amounts of each fraction from equivalent numbers of

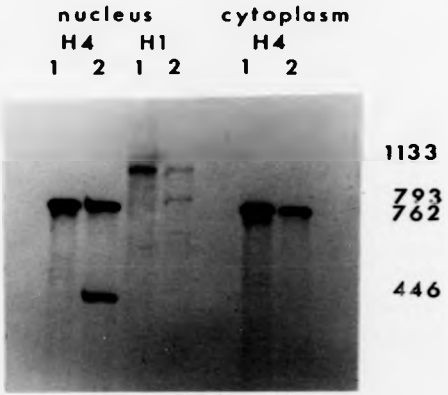
injected oocytes were then analysed by acrylamide gel electrophoresis, followed by autoradiography. As a control, to show that the oligo - dT assay had worked, a small amount of uninjected H4 precursor mRNA was adenylated *in vitro* using polyA polymerase. This adenylated H4 RNA was used in a parallel batch binding assay under identical conditions to the injected RNA samples. A small amount of uninjected H4 RNA, known to be non - adenylated, was also used in a control assay. The results of this experiment are shown in figure 16.

In the nuclear injection experiments both H4 and H1 precursor mRNAs were non - adenylated on injection and remained non - adenylated after 3 hours in the oocyte. The 3' cleaved H4 and H1 RNAs were also non - adenylated.

The H4 precursor mRNA injected into the oocyte cytoplasm was non - adenylated and remained non - adenylated after 3 hours in the oocyte. The control assays showed that the oligo - dT binding assay had worked. The majority of the artificially adenylated H4 precursor mRNA was in the polyadenylated fraction whereas none of the non - adenylated H4 RNA was in this fraction.

This experiment demonstrated that neither precursor or 3' cleaved H4 or H1 RNAs were polyadenylated as defined by the oligo - dT assay. However the possibility remained that the RNAs had short poly - A tails similar to those found on the 3' ends of endogenous oocyte histone mRNAs. The binding assay might not have been sensitive enough to fractionate on the basis of these short A tails. To investigate this, endogenous oocyte and matured oocyte RNAs were fractionated using the same oligo - dT assay.

Fig. 15


nucleus
H4
1 2
H1
1 2
cytoplasm
H4
1 2
1133
793
762
446

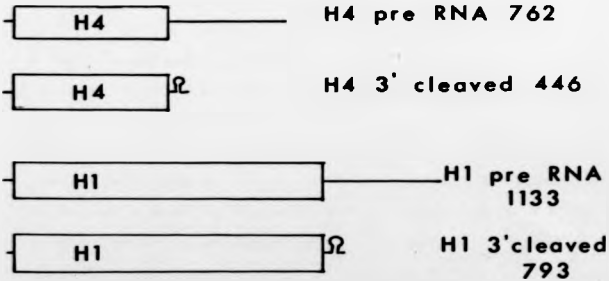

H4
H4 pre RNA 762
H4
H4 3' cleaved 446
H1
H1 pre RNA
1133
H1
H1 3'cleaved
793

Figure 15. Injection of H4 and H1 precursor mRNAs into the nucleus and cytoplasm of stage 6 Xenopus oocytes

Radioactively labelled H4 and H1 precursor RNAs were injected into either the nucleus or cytoplasm of stage 6 Xenopus oocytes. RNA samples were prepared from oocytes immediately after injection (1) and from oocytes 3 hours after injection (2). RNA samples from equal numbers of oocytes were analysed by polyacrylamide gel electrophoresis.

Nucleus H4 = RNA samples from H4 precursor mRNA nuclear injections.

Nucleus H1 = RNA samples from H1 precursor mRNA nuclear injections.

Cytoplasm H4 = RNA samples from H4 precursor mRNA cytoplasmic injections.

All sizes marked are in nucleotides.

Fig. 16

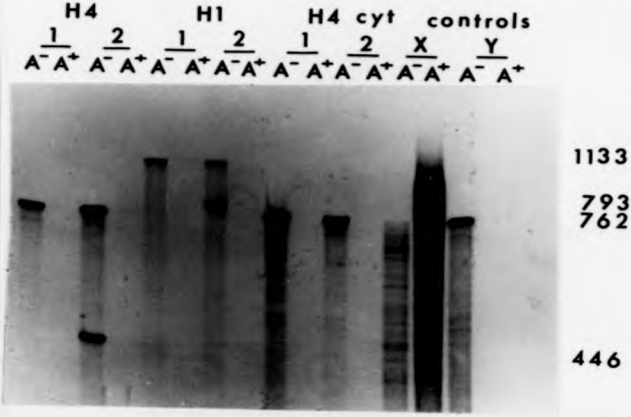
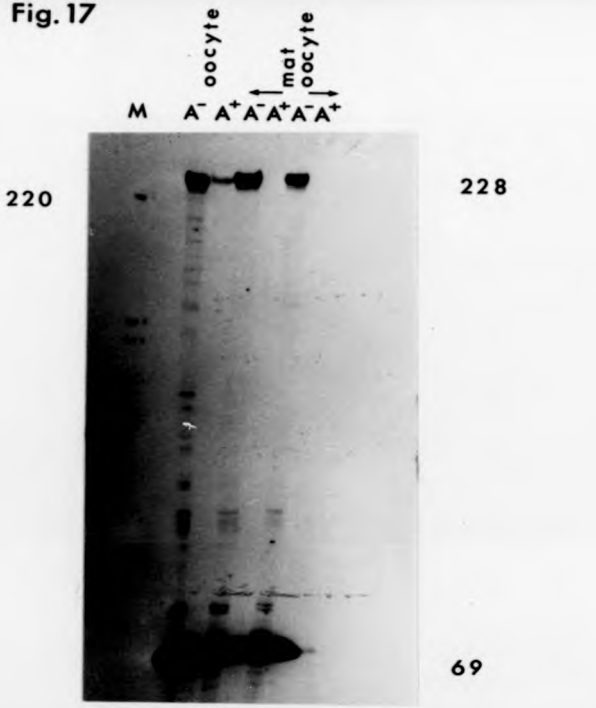

H4
H1
H4 cyt
controls
1
2
1
2
1
2
X
Y
A⁻ A⁺ A⁻ A⁺ A⁻ A⁺ A⁻ A⁺ A⁻ A⁺ A⁻ A⁺ A⁻ A⁺ A⁻ A⁺
1133
793
762
446

Figure 16. Oligo dT cellulose binding assays of H4 and H1 precursor mRNAs after injection into the stage 6 oocyte

Radioactively labelled H4 and H1 precursor mRNAs were injected into the nucleus or cytoplasm of stage 6 oocytes. RNA samples were prepared from oocytes immediately after injection (1) and from oocytes 3 hours after injection (2). The RNA samples were fractionated into adenylated (A+) and non - adenylated (A-) fractions using and oligo dT cellulose binding assay. The fractionated RNA samples were then analysed by acrylamide gel electrophoresis.

H4 = RNA samples from H4 precursor mRNA nuclear injections.
H1 = RNA samples from H1 precursor mRNA nuclear injections.
H4 cyt = RNA samples from H4 precursor mRNA cytoplasmic injections.
Control X = Samples from an oligo dT cellulose binding assay using artificially adenylated H4 precursor mRNA.
Control Y = Samples from an oligo dT cellulose binding assay using uninjected, non - adenylated, H4 precursor mRNA.
1133 nt = Size of H1 precursor mRNA.
793 nt = Size of 3' cleaved H1 RNA.
762 nt = Size of H4 precursor mRNA.
446 nt = Size of 3' cleaved H4 RNA

Fig. 17


oocyte
mat oocyte
M
A⁻ A⁺ A⁻ A⁺ A⁻ A⁺
220
228
69

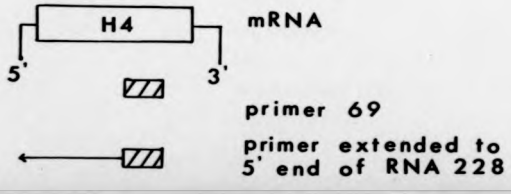

H4
mRNA
5'
3'
primer 69
primer extended to
5' end of RNA 228

Figure 17. Primer extension analysis of oocyte and matured oocyte adenylated and non - adenylated H4 transcripts

RNA samples prepared from stage 6 oocytes (oocyte) and from matured oocytes (mat oocyte) were fractionated into adenylated (A+) and non - adenylated (A-) fractions using an oligo dT cellulose batch binding assay. RNA fractions from stage 6 oocytes and duplicate RNA fractions from matured oocytes were then subjected to a primer extension analysis using a primer prepared from the H4 gene in pSP64.XlH4.

M = pAT153 Hinf I size marker.

Oocyte A- , oocyte A+ = Primer extension analysis on fractionated RNAs from stage 6 oocytes.

Mat oocyte A-, mat oocyte A+ (4 gel tracks, duplicate samples) = Primer extension analysis on fractionated RNAs from matured oocytes.

69 nt = Size of unextended primer.
228 nt = Size of primer extended to 5' end of H4 transcripts.

All sizes labelled are in nucleotides.

The location of the H4 histone mRNAs was determined using a primer extension assay. The primer was a 69 nt Hha I / Ava I fragment from the H4 gene in pSP64.XlH4. Extension of this primer to the 5' end of H4 transcripts gave extension products about 228 nt in length. Previous work (Ballantine and Woodland, 1985) has shown that the oocyte contains H4 transcripts with short poly - A tails whereas the matured oocyte does not contain these adenylated H4 transcripts. The result of the primer extension assay shown in figure 17 is consistent with these results. In the stage 6 oocyte, although the majority of H4 transcripts were found in the non - adenylated fraction, an easily detectable level of H4 transcripts were in the adenylated fraction. In the matured oocyte samples all the H4 transcripts were in the non - adenylated fraction, nothing was detected in the adenylated fraction. These results indicated that the oligo - dT assay was sufficiently sensitive to fractionate at least some of the H4 transcripts with short poly - A tails.

c. Discussion

The results presented in this chapter have confirmed that precursor mRNAs made from Xenopus H4 and H1 genes undergo size changes when injected into the nucleus of the oocyte. These size changes are consistent with them being the result of a cleavage reaction at the mRNA 3' end position. Each precursor mRNA had a cap at the 5' end and a small amount of polylinker sequence from the SP6 plasmid at the immediate 5' and 3' ends. The latter fact did not prevent the cleavage of the precursor mRNAs, this is

consistent with the finding that only the conserved sequences around the mRNA 3' end position and a certain amount of spacer sequence must be present in the precursor mRNA for processing to occur (Birchmier et al, 1982, 1983, 1984). The oocyte 3' processes the injected precursor mRNAs with high efficiency. The results suggest that approximately 50% of each precursor mRNA was 3' processed in the 3 hours of the experiment. However some of the remaining unprocessed RNA seen in these experiments was undoubtedly the result of injections that failed to hit the nucleus. The remaining precursor mRNA may not have been processed because some component of the processing machinery was limiting or because it was transported out of the nucleus before processing could occur. In these experiments approximately 2ng of precursor mRNA was injected per oocyte. Assuming that 50% of this was processed over the course of the experiment it indicates that the oocyte has the capacity to 3' process about 1ng of precursor mRNA in 3 hours. The stage 6 oocyte contains a steady state level of 130 pg of histone H3 mRNA (Van Dongen et al, 1981). Assuming that the other histone mRNAs are present at about the same abundance then each oocyte contains about 650 pg of histone mRNA, this is approximately the same amount of mRNA that would result from the amount of artificial precursor mRNA 3' processed in 3 hours. Each oocyte therefore has the ability to 3' process, what to it is a relatively large amount of histone precursor mRNA, in a short time. This result is fairly suprising because it has been shown that stage 6 Xenopus oocytes are relatively deficient in Sm precipitable U - snRNPs (Zeller et al, 1983).

This work suggested that the oocyte stockpiles the protein components of U snRNPs in the cytoplasm, these are then used by the embryo to complex with newly transcribed snRNAs later in development. This work also showed that the U1 and U2 snRNAs reach a maximal level early in oogenesis and decrease to about 20% of this value in the stage 6 oocyte. It has been shown that the sea urchin H3 3' processing requires the Sm precipitable U7 snRNP. If the oocyte histone processing snRNP (U7 equivalent) followed the general pattern established for the other U snRNPs then the oocyte might be expected to be relatively poor at 3' processing histone precursor mRNAs.

The results have also confirmed that H4 precursor mRNA injected into the oocyte cytoplasm is stable but does not undergo any 3' processing; in the oocyte the processing machinery is restricted to the nucleus.

The investigation of the polyadenylation status of the artificial RNAs showed that neither the precursors or the 3' cleaved RNAs became polyadenylated in either the nucleus or the cytoplasm of the stage 6 oocyte. The investigation of the polyadenylation status of the oocytes own histone H4 transcripts was in agreement with previously published work (Ballantine and Woodland, 1985). Polyadenylated H4 transcripts were detectable in the oocyte and were absent from the matured oocyte. The percentage of H4 transcripts that bound to oligo - dT cellulose in my experiments was less than previously reported, this may be due to some variation between individual animals or it may be a reflection on the oligo - dT cellulose binding procedures used.

The A tracts on the oocyte histone transcripts are very short yet the binding assay was sensitive enough to detect them. If the mRNA 3' ends generated from the artificial precursor mRNAs had shown the same pattern of polyadenylation as the endogenous histone mRNAs then at least a detectable proportion of them would have bound to oligo - dT cellulose. Previous workers have found that although the stage 6 oocyte contains adenylated histone transcripts it does not polyadenylate histone mRNA 3' ends made from injected DNAs, this was shown for the sea urchin H2A and H2B genes (Probst et al, 1979), the chicken H2B gene (Krieg and Melton, 1984) and the chicken H5 gene (Wigley et al, 1985). The results presented in this chapter have shown that the same is true for a Xenopus H4 mRNA 3' end made from an artificial precursor mRNA, where that precursor was made from a gene known to contribute polyadenylated transcripts to the oocyte. This is a reflection on the way in which the oocyte makes polyadenylated histone transcripts. These transcripts are probably made from precursor mRNAs by a 3' cleavage reaction in the usual way. Polyadenylation is not linked to 3' cleavage but occurs after the mRNA 3' end has been formed. The polyadenylation activity has possibly been lost by the time the oocyte reaches stage 6 but polyadenylated histone transcripts made earlier in oogenesis persist.

3.2 3' CLEAVAGE OF HISTONE PRECURSOR mRNAs IN MATURED OOCYTES, EGGS AND EMBRYOS

a. Introduction

These experiments were carried out to discover whether stages in Xenopus development between the stage 6 oocyte and the mid - blastula embryo were able to 3' cleave histone precursor mRNAs. Any model that invokes 3' cleavage of stored histone precursor mRNAs, leading to their translation in the early embryo, would require the presence of 3' processing mechanisms in these stages. On the other hand there is no new transcription in the early embryo until the mid - blastula transition (MBT) (Newport and Kirschner, 1982a; 1982b) so it is possible that the early embryo does not possess mRNA 3' processing mechanisms until the MBT.

A histone H4 precursor mRNA, previously shown to be 3' cleaved in the oocyte nucleus, was tested for cleavage in matured oocytes, eggs and embryos.

b. Results

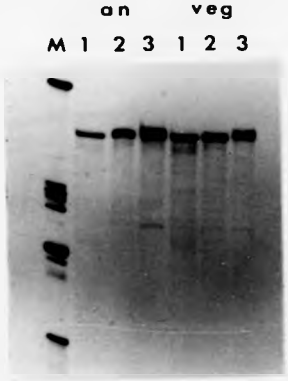
A H4 precursor mRNA was transcribed *in vitro* from the pSP64.XlH4 sub - clone. A cap analogue and a labelled nucleotide were included in the mix to produce a capped and labelled precursor mRNA. This precursor mRNA was injected into either matured oocytes, eggs or early embryos. Mature oocytes were obtained by treating stage 6 oocytes with progesterone. On

maturation the germinal vesicle breaks down and a white spot appears at the animal pole. Only oocytes with a clearly visible white spot were regarded as completely matured and suitable for injection. Unfertilised eggs were obtained from hormonally stimulated females. The eggs were treated with cysteine to remove the jelly layer prior to injection. Embryos were obtained by fertilising eggs *in vitro*, they were allowed to develop to the first cleavage stage before injection. One cell of each two cell embryo was injected. Approximately 30 eggs or embryos were injected in the animal hemisphere (unless stated otherwise). Samples were taken immediately after injection and at two further time points. For the embryo injections the time points were 0, 3 and 6 hours after injection. The last time point was selected to be approximately 2 hours after the MBT so that any processing changes at this transition could be detected. The same time points were used in the matured oocyte and egg injections so they were comparable. On injection both matured oocytes and unfertilised eggs were activated, they began a series of abortive cleavages which would eventually lead to death. Over the incubation periods used in these experiments the majority of injected matured oocytes and eggs remained healthy, as judged by their external appearance. Only healthy matured oocytes or eggs were used to prepare RNA samples. The RNA samples from the different time points were analysed directly by acrylamide gel electrophoresis. In each case RNA from an equal number of oocytes or embryos was loaded onto each track of the gel.

Fig. 18

Injection of pSP64.H4 RNA

A. matured oocytes



B. unfertilised eggs

Figure 18. Injection of H4 precursor mRNA into matured oocytes and unfertilised eggs

Radioactively labelled H4 precursor mRNA was injected either into matured oocytes or unfertilised eggs. RNA samples were prepared from oocytes or eggs immediately after injection (1), 3 hours after injection (2) and 6 hours after injection (3). These RNA samples were analysed by acrylamide gel electrophoresis.

Figure 18.A. Injection of H4 precursor mRNA into matured oocytes. Matured oocytes were injected into either the animal pole (an) or vegetal pole (veg). RNA samples from the 3 time points described above were analysed by acrylamide gel electrophoresis.

M = pBR322 Hinf I digest size marker.
762 nt = Size of H4 precursor mRNA.
446 nt = Size of 3' cleaved H4 RNA.

Figure 18.B. Injection of H4 precursor mRNA into unfertilised eggs. RNA samples from the 3 time points described above were analysed by acrylamide gel electrophoresis

762 nt = Size of H4 precursor mRNA.
446 nt = Size of 3' cleaved H4 RNA.

Fig. 19
Injection of H4 and MG.1 RNAs into Xenopus embryos

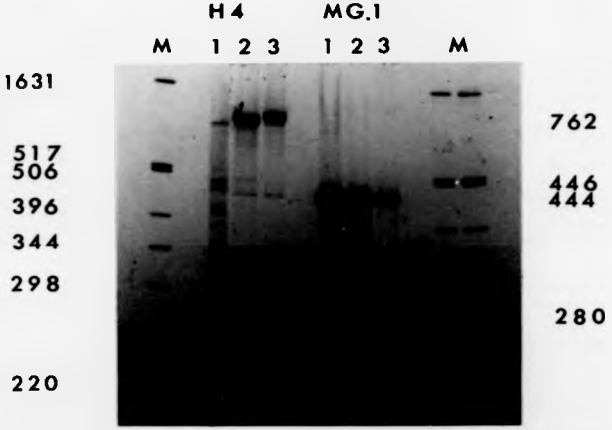


Figure 19. Injection of H4 precursor mRNA into Xenopus embryos

Radioactively labelled H4 and MG1 precursor mRNAs were injected into Xenopus embryos at the first cleavage stage. RNA samples were prepared from embryos immediately after injection (1), 3 hours after injection (2) and 6 hours after injection (3). These RNA samples were analysed by acrylamide gel electrophoresis. 3 hours after injection = st. 7

6 hours after injection = st. 9

M = pBR322 Hinf I digest size marker.

H4 1, 2, 3 = Samples from embryos injected with H4 precursor mRNA.

MG1 1, 2, 3 = Samples from embryos injected with MG1 precursor mRNA.

762 nt = Size of H4 precursor mRNA.

446 nt = Size of 3' cleaved H4 RNA.

444 nt = Size of MG1 precursor mRNA.

280 nt = Size of 3' cleaved MG1 RNA.

All sizes marked are in nucleotides.

Figure 18 shows the results of the matured oocyte and egg injections. Figure 18 A is the matured oocyte result, here injections were either into the animal pole or the vegetal pole of the matured oocyte. On injection into the animal pole 2 effects were seen. The first effect was that over the course of the experiment a small amount of the 762 nt precursor mRNA was cleaved at the mRNA 3' end position to produce the 446 nt RNA. The 3' cleavage reaction was inefficient compared to the same reaction in the oocyte nucleus. The majority of the H4 precursor mRNA did not undergo 3' cleavage. The second effect involved the unprocessed H4 precursor mRNA. The precursor RNA band broadened considerably over the course of the experiment due to the conversion of the precursor mRNA to a slower migrating form. On injection into the vegetal pole the same two effects were seen.

Figure 18 B Shows the result of injections into unfertilised eggs, these showed the same results as the matured oocyte injections. 3' cleavage of the precursor mRNA ocurred but this was inefficient. Conversion of the precursor mRNA to a slower migrating form also ocurred.

Figure 19 shows the result of the embryo injections. Although the loadings on this gel were not quite right it demonstrates clearly that 3' cleavage of injected H4 precursor mRNA occured in the Xenopus embryo. The faint band at 446 nt was caused by histone RNA cleaved at the normal 3' end position. This 3' cleavage reaction was relatively inefficient. The 446 nt band is about the same intensity in the 2nd and 3rd time point samples, this indicates that most of the 3' cleavage that could

occur took place relatively quickly after injection. There were no dramatic changes in RNA processing after the MBT. The second effect observed from the matured oocyte and egg injections was also observed here. The unprocessed H4 precursor mRNA underwent conversion to a slower migrating form. The other 3 tracks on this gel show the result of the same experiment using a mouse globin precursor mRNA transcribed from pSP64.MG1 (see figure 3). This precursor mRNA did not undergo 3' cleavage in the embryo and neither did it undergo conversion to a slower migrating form.

c. Discussion

The results have shown that a H4 precursor mRNA microinjected into matured oocytes, eggs or embryos underwent inefficient but accurate 3' cleavage. Matured oocytes have undergone germinal vesicle breakdown with the result that there is no defined nucleus. The 3' cleavage observed in these injections is therefore a non - nuclear processing reaction. By comparison in the stage 6 oocyte the histone mRNA 3' cleavage activity is restricted to the nucleus. Two sites of injection: the animal pole and the vegetal pole were used in these injections. This was to counter the argument that the small amount of 3' cleavage detected was the result of inadvertent nuclear injection of a non matured oocyte. By injecting into the vegetal pole it would have been very difficult accidentally to hit the nucleus if the oocyte was not properly matured. Although the processing reaction is non - nuclear it is probably still mediated by components of the nuclear RNA processing machinery,

distributed around the cell after germinal vesicle breakdown. However the possibility that a new cytoplasmic RNA processing activity was involved cannot be completely ruled out.

Unfertilised eggs gave the same result as the matured oocytes, this was not suprising because a stage 6 oocyte matured *in vitro* and an unfertilised egg can be regarded as largely equivalent to each other.

It is impossible to be certain as to the location of the 3' cleavage activity detected in the early embryos. Some of the embryos might have been injected in a cell nucleus or precursor mRNA might have been incorporated into the embryonic nuclei during the rapid early cleavages of embryogenesis. Alternatively 3' cleavage might have been entirely cytoplasmic.

The fact that these early developmental stages have the ability to 3' cleave injected histone precursor mRNAs does not necessarily mean that it is physiologically significant. As already suggested it is possible that the activity is merely the result of germinal vesicle breakdown distributing the nuclear contents around the matured oocyte or unfertilised egg. However the detection of the activity, in a situation where it would not be needed to process newly transcribed histone precursor mRNAs, means that the model which involves storage of precursor mRNAs in the oocyte followed by their 3' processing and translation on fertilisation could operate.

To investigate further such a possibility experiments were carried out to determine whether histone precursor mRNAs could be detected in the oocyte (see below).

The results also show a previously undescribed effect. Much of the injected precursor mRNA did not undergo 3' cleavage, it was converted to a form which migrated more slowly on electrophoresis. This effect was seen in microinjected matured oocytes, eggs and embryos but was absent over similar incubation times in the nucleus or cytoplasm of the stage 6 oocyte (see section 3.1). One possible explanation for the effect was that the uncleaved RNA became polyadenylated, this made it increase in length and so it migrated more slowly on electrophoresis. This idea was tested as described later (see section 3.3). After injection of the precursor mRNA most of the 3' cleavage that occured took place soon after injection whereas the conversion to the slower migrating form took longer. The possibility that the conversion of the precursor to this different form inhibited 3' processing was tested as described later (see section 3.3).

Do histone precursor mRNAs exist in the oocyte ?

Having established that microinjected eggs and embryos can 3' cleave histone precursor mRNAs an attempt was made to identify such precursors in the stage 6 oocyte and the egg. If the processing activity was present for the reasons suggested then large amounts of histone precursor mRNAs should be stored in the oocyte.

Total RNA was prepared from stage 6 oocytes and from eggs. Samples of each were were electrophoresed on an agarose gel. The gel was photographed to show the position of the ribosomal RNA

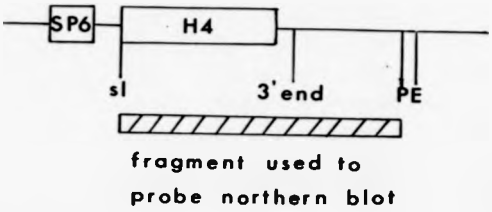
bands and was then blotted onto nitrocellulose using the Northern blotting procedure. The nitrocellulose filter was probed with a nick - translated DNA probe. The probe consisted of the H4 sequence from pSP64.XlH4, this was the H4 gene for which a 3' cleavage activity had been demonstrated in eggs and embryos. The probed filter was washed and autoradiographed to give the result shown in figure 20. The thick band at about 362 nt was caused by hybridisation of the probe to H4 transcripts from a number of H4 genes, this band was similar in size and intensity in both oocyte and egg samples. In neither case was it possible to detect an RNA band larger in size than the mature H4 mRNA. A similar experiment has been carried out using a Xenopus H3 histone gene probe (Old, personal communication), again histone transcripts that might have been precursor mRNAs were not detected.

Histone precursor mRNAs, of any size appreciably longer than mature H4 mRNAs, could not be detected in the stage 6 oocyte or in the egg. The 3' cleavage activity detected in microinjected eggs and early embryos would not therefore be required for the processing of a significant amount of stored precursor mRNA. The activation of the histone mRNA store is probably not achieved by the mechanism of 3' cleavage of unprocessed precursor mRNAs. Other mechanisms such as the sequestration of histone mRNA in the oocyte and its availabilty in the early embryo, modifications to the cap structure or the change in polyadenylation status of histone mRNAs are likely to be involved.

Fig. 20

A.

pSP64.XIH4



B.

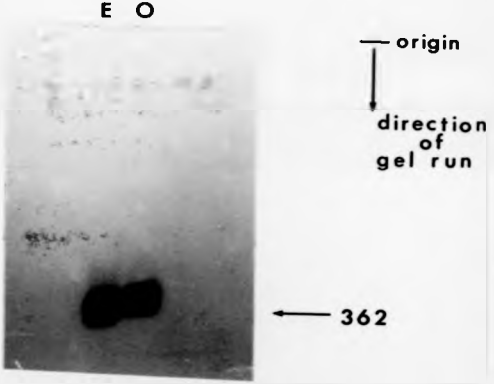


Figure 20. Northern analysis of RNA from oocytes and eggs using a histone H4 probe

Total RNA prepared from Xenopus oocytes and unfertilised eggs was electrophoresed on an agarose gel. The RNA samples were blotted onto nitrocellulose using the Northern blotting procedure. The nitrocellulose was then probed with a radioactively labelled histone H4 DNA probe.

Figure 20.A. The pSP64.XlH4 sub - clone used to prepare the radioactive probe. The Sal I / Pst I fragment underlined was isolated and radioactively labelled then used to probe the nitrocellulose filter.

Key to abbreviations of restriction enzyme sites :

Sl = Sal I P = Pst I E = Eco RI

3' end = Histone H4 mRNA 3' end position.

Figure 20.B. Autoradiograph of the nitrocellulose filter probed with the H4 probe.

E = Sample of RNA prepared from unfertilised eggs.

O = Sample of RNA prepared from oocytes.

362 nt = Size of H4 mRNA.

3.3 A POLYADENYLATION ACTIVITY IN MATURED OOCYTES, EGGS AND EMBRYOS

a. Introduction

It was observed that an H4 precursor mRNA microinjected into matured oocytes, eggs and embryos was inefficiently 3' cleaved. The remaining precursor mRNA was subject to a second reaction which converted it to a form that migrated more slowly on acrylamide gel electrophoresis. One obvious explanation for this effect was that the RNA was polyadenylated in these stages, this would have made it larger in size and hence decreased its electrophoretic mobility. In this chapter experiments to test this explanation are described.

It was also observed that in these microinjection experiments 3' cleavage occured soon after injection whereas the conversion of the RNA form occured more slowly, this gave rise to the suggestion that the conversion of the precursor mRNA, to the slower migrating form, inhibited 3' cleavage. Experiments to test this idea are also described.

b. Results

A capped and labelled H4 precursor mRNA was made from pSP64.X1H4 (see figure 12) and injected into matured oocytes as previously described. RNA samples from injected matured oocytes were prepared from matured oocytes immediately after injection and from matured oocytes three hours after injection.

RNA samples from an equal number of matured oocytes, from the two time points, were bound to oligo - dT cellulose. Adenylated and non - adenylated RNA fractions from each time point sample were obtained. These fractions were then analysed directly by acrylamide gel electrophoresis followed by autoradiography. Each sample loaded onto the gel contained an amount of adenylated or non - adenylated RNA from an equal number of oocytes. By autoradiography of the gel the polyadenylation status of the labelled H4 RNAs was determined. A similar experiment was carried out using a labelled H1 precursor mRNA transcribed from pSP64.X1H1 (see figure 11).

Figure 21.A shows 2 duplicate assays for the H4 injection experiment. Tracks 1 and 2 contained adenylated and non - adenylated RNA from the sample taken immediately after injection. The 762 nt precursor mRNA band is only in the non - adenylated fraction, this was expected because the precursor mRNA was not poladenylated prior to injection. Tracks 3 and 4 contained adenylated and non - adenylated RNA from the sample taken 3 hours after injection. In this case the non - adenylated fraction contains some of the uncleaved precursor mRNA and all of the shorter (446 nt) RNA produced as a result of 3' cleavage. Now however the adenylated fraction also clearly contains some of the precursor mRNA. The precursor mRNA in the adenylated fraction has migrated more slowly on electrophoresis than the equivalent RNA in the non - adenylated fraction, this was due to the addition of the poly - A tail increasing the length of the precursor mRNA. These results have shown that the conversion of the H4 precursor

mRNA to a slower migrating form (described in section 3.2) was due to a polyadenylation activity using the precursor mRNA as a substrate. Approximately 50% of the injected H4 precursor mRNA was polyadenylated over the 3 hours of the experiment.

Figure 21.B shows the result from the H1 injection experiment. The H1 bands are faint on this autoradiograph, this was because the size of the H1 precursor mRNA caused it to run in approximately the same position as the oocyte ribosomal RNA on electrophoresis. For this reason if anything other than a very small amount of RNA, from injected oocytes, was loaded the H1 band smeared. Because the H1 bands are faint it is not possible to see the small amount of 3' cleaved H1 RNA on this photograph. It does however demonstrate that the H1 precursor mRNA was also polyadenylated after injection into matured oocytes. In this case virtually all of the injected H1 precursor mRNA was polyadenylated, this was probably due to the batch of matured oocytes used being particularly efficient at polyadenylation. In these oocytes virtually all of the injected H4 precursor mRNA was polyadenylated.

This oligo - dT binding assay was only used for samples from injected matured oocytes. It was assumed that in injected eggs and embryos, where the same effect was observed, the same polyadenylation activity was responsible.

Having established that the slower migrating RNA form in these experiments was due to polyadenylation of the precursor mRNA,experiments were carried out to determine if polyadenylation of precursor mRNA inhibited 3' cleavage, this was in order to see

whether the polyadenylation activity, observed in injected matured oocytes, was responsible for the low cleavage efficiency. Capped, labelled H4 precursor mRNA transcribed from pSP64.XlH4 was polyadenylated *in vitro* using E.coli poly - A polymerase. This enzyme adds A tracts of various lengths to the 3' ends of RNA molecules. The average length of the A tract added depends on the reaction time. The polyadenylated H4 precursor mRNA was tested for 3' cleavage in the nucleus of the stage 6 oocyte. The results are shown in figure 22. The tracks on the left of the gel show the adenylated and the non - adenylated precursor mRNAs prior to injection. Poly - A polymerase treatment has converted the sharp H4 RNA band to a smear by adding poly A tails of disparate lengths. On injection into the oocyte nucleus the polyadenylated H4 precursor mRNA was 3' cleaved as efficiently as the non - adenylated precursor, both were converted to the 446 nt RNA consistent with cleavage at the mRNA 3' end position. It was concluded that in the oocyte nucleus the presence of a 3' poly - A tail does not prevent cleavage of a precursor mRNA

c. Discussion

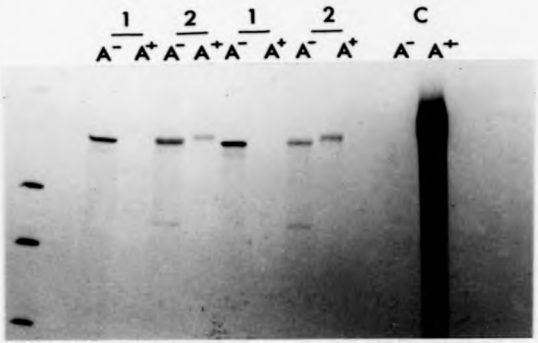
The results presented in this section have shown that microinjected matured oocytes, eggs and early embryos of Xenopus laevis contain a polyadenylation activity. This activity is not present in the nucleus or the cytoplasm of the stage 6 oocyte (see figure 16 for example). Both microinjected H4 and H1 precursors mRNAs act as a substrate for this activity. It could possibly work in two ways: either the poly - A tract could be

added directly to the end of the precursor mRNA or there could be a 3' cleavage near to the end of the precursor mRNA followed by the addition of a long poly - A tract causing a nett increase in size. Whichever the correct explanation the polyadenylation reaction is controlled, the A tails added are all of a similar length because the polyadenylated precursor mRNAs run as a tight band rather than as a smear on gel electrophoresis (see figure 21). Because of the controlled nature of the polyadenylation reaction the possibility that the H4 and H1 precursor mRNAs contain sequences located near their 3' ends which direct the activity was considered. Both precursor mRNAs contain 36 nt of SP6 polylinker directly at the 3' end. The sequence adjoining this was not available for the H4 gene. The H1 sequence was examined (see figure 23). It was observed that the polyadenylation hexamer AATAAA was located 24 nt upstream from the Pst I site at the end of the H1 fragment in pSP64.X1H1, this put the hexamer at a position about 60 nt upstream from the 3' end of the precursor mRNA. It was considered possible that this sequence directed the polyadenylation of the precursor in certain circumstances. At present the precise role of the AATAAA sequence in polyadenylation is not clear. It is known to be of prime importance in directing the 3' cleavage reaction which is normally followed by polyadenylation. However there is evidence that it also directs polyadenylation in the absence of 3' cleavage (Manley, 1983, Manley et al,1985, Zarkower et al, 1986). A more detailed investigation of the polyadenylation activity was carried out as described in section 3.4.

Fig. 21

A.

pSP64.XIH4



B.

pSP64.XIH1

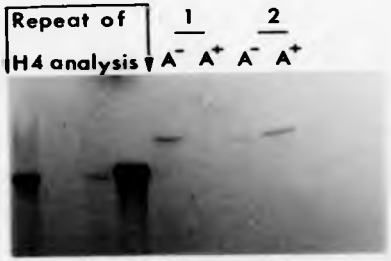


Figure 21. Oligo dT cellulose binding assay on H4 and H1 precursor mRNAs after injection into matured oocytes

Radioactively labelled H4 and H1 precursor mRNAs were injected into matured Xenopus oocytes. RNA samples were prepared from oocytes immediately after injection (1) and from oocytes 3 hours after injection (2). These RNA samples were fractionated into adenylated (A+) and non - adenylated (A-) fractions using an oligo dT cellulose batch binding assay. The RNA fractions were then analysed directly by acrylamide gel electrophoresis.

Figure 21.A. Analysis of H4 precursor mRNA injections. This gel shows 2 duplicate analyses.

Figure 21.B. Analysis of H1 precursor mRNA injections.

1 A- and 1 A+ = Adenylated and non - adenylated RNA samples from oocytes immediately after injection.
2 A- and 2 A+ = Adenylated and non - adenylated RNA samples from oocytes 3 hours after injection.
C A- and C A+ = Adenylated and non - adenylated RNA samples from a control assay using adenylated H4 precursor mRNA.

762 nt = Size of H4 precursor mRNA 446 nt = Size of 3' cleaved H4 RNA. 1133 nt - Size of H1 precursor mRNA.

Fig. 22

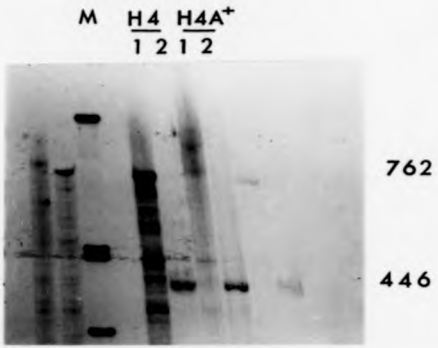


Figure 22. 3' cleavage of polyadenylated H4 precursor mRNA injected into the oocyte nucleus

Radioactively labelled H4 precursor mRNA was polyadenylated *in vitro*. Polyadenylated H4 precursor mRNA was then injected into the oocyte nucleus and RNA samples were prepared from oocytes immediately after injection (1) and from oocytes 3 hours after injection (2). The RNA samples were analysed by acrylamide gel electrophoresis.

M = pAT153 Hinf I size marker.
H4 1 and H4 2 = RNA samples from oocytes injected with non - adenylated H4 precursor mRNA.
H4A+ 1 and H4A+ 2 = RNA samples from oocytes injected with H4 precursor mRNA adenylated *in vitro* prior to injection.

762 nt = size of H4 precursor mRNA (before addition of the A tail).
446 nt = size of 3' cleaved H4 RNA.

All sizes labelled are in nucleotides.

Fig. 23

5'

CCTCCGCTAA GAAAGTTCAG TCTAATAGCC GCGTTTCAAC TATTCATTTA AGGGCCGATT

TTGCCGATGT AATAAAGAGT TTCTATTTAA CCACTTCAAC TGCAGGTCGA CTCTAGAGGA

------ 3'

TCCCGGGGCG AGCTCG AATT C

Pst I

Eco RI

(Sequence from Turner et al, 1983)

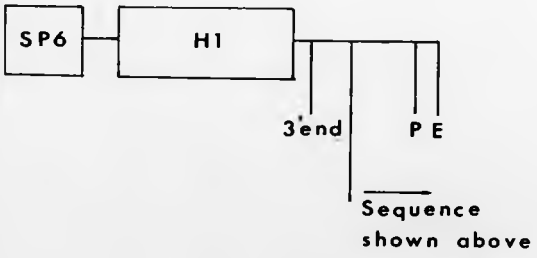


Figure 23. DNA sequence of the 3' end of the H1 fragment cloned in pSP64.X1H1

The DNA sequence was obtained from Turner et al, 1983. The sequence represents the 3' 100 nt of the H1 histone fragment cloned in pSP64.X1H1. The 3' end of this fragment was at a Pst I restriction enzyme site (Pst I). The sequence downstream of this site to the Eco RI (Eco RI) site, used to linearise the clone for SP6 transcription, is also shown as this extra polylinker sequence was present in H1 SP6 precursor mRNA.
The AATAAA sequence (underlined) is located approximately 20 nt upstream from the 3' end of the H1 fragment and was located approximately 60 nt upstream from the 3' end of H1 precursor mRNA.

Key to abbreviations of restriction enzyme sites :

P = Pst I E = Eco RI

3' end = H1 mRNA 3' end position.

The 3' cleaved H4 RNA produced in matured oocytes was not polyadenylated, this might have been because the cleavage reaction removed downstream sequences which in the precursor mRNA directed polyadenylation. Alternatively the 3' cleaved H4 RNA may have been inaccessible to the polyadenylation activity, for example it may have remained associated with the 3' cleavage snRNPs.

It was also shown that a polyadenylated H4 precursor mRNA was 3' cleaved when injected into the oocyte nucleus. This 3' cleavage was as efficient as when a non - adenylated precursor mRNA was injected. It has already been shown that histone precursor mRNAs can be 3' cleaved as long as the sequences around the mRNA 3' end position and a certain amount of spacer sequence are present (Birchmier et al, 1982, 1983, 1984). The length of extension at the 3' end does not seem to be important as long as these sequences are included. Adding an A tail involves, in effect, increasing the length of the 3' extension by adding A residues. Based on available evidence it was not suprising that this did not inhibit 3' cleavage. One possibility was that adding the A tail to the precursor mRNA might have caused it to be exported from the nucleus before 3' cleavage could occur. This does not seem to have been the case. The finding that in the oocyte nucleus poladenylation does not inhibit 3' cleavage suggests that in matured oocytes the conversion of the precursor to the adenylated form was not responsible for the inefficient 3' cleavage observed.

3.4 FURTHER INVESTIGATION OF THE POLYADENYLATION ACTIVITY IN MATURED OOCYTES

a. Introduction

It has been demonstrated that artificial H4 and H1 precursor mRNAs are polyadenylated when microinjected into matured oocytes of *Xenopus laevis*. Experiments were carried out to investigate further this previously undescribed polyadenylation activity. Both the mechanism of the activity and the developmental stage at which it appears were studied.

It was considered likely that the activity worked in one of two ways : either the poly - A tail was added directly to the end of the precursor mRNA or there was a 3' cleavage close to the end of the precursor mRNA prior to polyadenylation. Microinjection experiments ,using a H1 precursor mRNA, were carried out to distinguish between these two possibilities.

From the histone precursor mRNA injection experiments it appeared that the activity was controlled, adding poly - A tails of a relatively constant length to each precursor mRNA molecule (see section 3.3, figure 21). The H1 precursor mRNA, shown to be a substrate for the activity, contained a copy of the polyadenylation hexamer AAUAAA 60 nt upstream from the 3' end of the precursor mRNA. This observation gave rise to the suggestion that this hexamer controlled the polyadenylation activity. The sequence of the H4 gene near to the 3' end of the precursor mRNA

was obtained. The H4 downstream sequence was then examined to see if the H4 precursor mRNA also contained a copy of the AAUAAA hexamer, if it did the idea that this sequence was involved in controlling the polyadenylation activity would have gained some support. To investigate further the possible sequence requirements of the activity 2 more precursor mRNAs were made from non - histone genes. These precursor mRNAs were then tested for polyadenylation in microinjected matured oocytes. If the activity was found to be general, using all injected RNAs as substrates, then it would be unlikely to be controlled by a specific sequence. Alternatively if the activity was found to polyadenylate certain RNAs and not others then a controlling sequence, such as the AAUAAA hexamer would be a possibility.

Finally the significance of the activity in relation to Xenopus early development was considered. The polyadenylation activity was shown to exist in microinjected matured oocytes but not in stage 6 oocytes (compare figure 21 with figure 16), this suggested that the activity was switched on when the oocyte matured into an egg. However on injection matured oocytes are activated, they begin a series of cleavages as if they had been fertilised, which eventually lead to the death of the oocyte. Therefore the possibility existed that the activity appeared as a result of activation and would not have been present in a non - activated matured oocyte. This was considered an important point for speculations as to the significance of the activity. After fertilisation of Xenopus eggs there is a rapid increase in the concentration of calcium ions in the egg cytoplasm (Balanger and

Schautz, 1975, Schroeder and Strickland, 1974). This increase is an essential step in the activation of the egg. The increase in free calcium ions can be prevented, in microinjected matured oocytes or eggs, by injecting EGTA along with the required sample. The EGTA chelates calcium ions and prevents activation. Experiments were carried out in which H4 precursor mRNA and EGTA were coinjected into matured oocytes to determine whether blocking activation blocked the polyadenylation activity. The analysis was slightly complicated because it was thought that removing calcium ions as well as preventing activation might have inhibited the enzyme responsible for the polyadenylation activity. As a control for this the experiment was repeated in early embryos. If the activity appeared as a result of activation and did not require calcium ions as an essential cofactor it was expected that coinjecting EGTA into matured oocytes would inhibit polyadenylation whereas coinjecting EGTA into embryos would have no affect. Alternatively if the EGTA blocked polyadenylation by removing a cofactor, in this case calcium ions, it would be equally inhibitory in matured oocytes and early embryos. Microinjection experiments were performed to investigate this point.

b. Results

1. Mechanism of polyadenylation

A capped, labelled H1 precursor mRNA, previously shown to be a substrate for the polyadenylation activity in matured oocytes,

was synthesised by transcription of pSP64.X1H1 (see figure 11). The H1 precursor mRNA was microinjected into matured oocytes as previously described. RNA samples were prepared from oocytes immediately after injection and from oocytes five hours after injection. Both RNA samples were fractionated into adenylated and non - adenylated fractions using oligo - dT cellulose. The position of the 3' end of the precursor mRNA in each fraction was then determined, in order to see whether the H1 precursor mRNA in the adenylated fraction was 3' cleaved prior to polyadenylation. To map the precursor mRNA 3' end an unlabelled DNA probe homologous to the RNA sequence, but not to any poly - A tail added, was used in a S1 nuclease protection assay. The probe used was a 603 nt Bgl I / Pvu II fragment prepared from pSP64.X1H1 (see figure 24.B). This probe contained 431 nt of sequence up to the Eco RI site where the plasmid was linearised for transcription and which was the end of the precursor mRNA. The unlabelled probe also contained 172 nt of sequence past this position. On hybridisation to untreated H1 precursor mRNA, followed by S1 nuclease treatment, this probe gave a protected fragment of 431 nt. Hybridisation to a 3' cleaved H1 precursor mRNA would have given a shorter protected fragment than this, the length dependent on the distance between the Bgl I site at the 5' end of the probe and the site of cleavage of the precursor mRNA. The results of a series of test hybridisations are shown in figure 24.A. The substrate used was uninjected H1 precursor mRNA, 1133 nt in length. The hybridisations were carried out at 3 different temperatures and at two S1 nuclease concentrations per

temperature. Each analysis contained an equal amount of RNA. After S1 nuclease treatment the protected RNA fragments were analysed by polyacrylamide gel electrophoresis. The S1 nuclease concentrations used were not critical but there were differences between the 3 temperatures. At 40°C and 50°C the labelled 1133 nt precursor mRNA gave a protected fragment of 431 nt when the probe was present, this was the size of protected fragment expected. At 60°C no protected fragments were seen, this temperature was too high for the probe to form stable hybrids with the RNA, so none of the RNA was protected from S1 nuclease treatment. Two controls were carried out, both at 50°C. When no probe was included none of the H1 precursor mRNA was protected from S1 nuclease digestion. When there was no probe and no S1 nuclease treatment the H1 precursor mRNA survived intact at a size of 1133 nt.

The probe was then used on the fractionated RNA from the H1 matured oocyte injections. A temperature of 50°C was chosen for the hybridisations which each contained an amount of adenylated or non - adenylated RNA from an equal number of injected matured oocytes. The results are shown in figure 24.B. Track C was a control using uninjected H1 RNA, this gave the 431 nt protected fragment. The 2 tracks which contained the non - adenylated RNAs from the 2 time points both contained the same 431 nt band caused by protection of full length H1 precursor mRNA, this band was fainter in the 5 hour sample because some of the RNA had been converted to the adenylated form. At the first time point no protected band was visible in the adenylated RNA fraction, obviously because immediately after injection all the H1 RNA was

non - adenylated. At the second time point a strong band was seen in the adenylated fraction, this was due to the conversion of the H1 precursor mRNA to the polyadenylated form. The size of this band was the same as the size of the non - polyadenylated RNA bands, within the limit of resolution of the gel. The 2 marker tracks give some idea of the limit of resolution in this region of the gel. Although one of the markers is faint it is possible to see a clear difference between the 527 /517 and the 404 /396 bands. Therefore within 5 - 10 nt the polyadenylated H1 3' end is in the same position as the 3' end of the non - adenylated precursor mRNA, this strongly suggests that 3' cleavage prior to polyadenylation does not occur.

2. Sequence specificity ?

The H4 gene in pSP64.XlH4 was partially sequenced in order to obtain the sequence proximal to the H4 precursor mRNA 3' end. This sequence was then examined for the presence of the AATAAA hexamer and compared with the sequence of the 3' end of the H1 precursor mRNA. The sequencing strategy used is shown in figure 25. The pSP64.XlH4 plasmid was linearised using Bam HI and then end - labelled using Klenow polymerase and α ^{32}P dGTP. The Bam HI site is located just upstream of the Eco RI site used when the plasmid was linearised for SP6 transcription (see figure 1). The labelled fragment was further digested using Ava I to give a 450 nt fragment labelled at one end only for sequencing. This fragment was treated to the Maxam and Gilbert sequencing reactions and the products were analysed on 8% and 20%

polyacrylamide gels (figure 26). In this way the sequence from the labelled Bam HI site in the SP6 polylinker into the downstream sequence of the H4 gene was obtained (Figure 27) . 120 nt of sequence from the 3' end of the H4 precursor mRNA is shown, this comprises 100 nt of sequence from the H4 gene and 20 nt of SP6 polylinker sequence. No exact match to the AATAAA hexamer was found, however the sequence AATAGA was identified about 95 nt upstream of the precursor mRNA 3' end position and the sequence AGTAAA was identified about 115 nt upstream of the precursor mRNA 3' end position (see discussion).

To investigate whether the activity would use any injected precursor mRNA as a substrate or whether it would only use certain RNAs, two non - histone precursor mRNAs were made and tested for polyadenylation in matured oocytes. The sub - clones pSP64.MG1 and pSP65.HS1 (see figures 28 and 29) were linearised with Eco RI and Hind III respectively. pSP64.MG1 contained a 392 nt fragment spanning the mRNA 3' end position of the mouse β globin gene. On transcription it gave a 444 nt precursor mRNA. It also gave rise to 2 smaller RNAs of about 250 and 220 nt, presumably as a result of premature transcription termination by the SP6 polymerase (see figure 13). pSP65.HS1 contained a 1160 nt fragment spanning the mRNA 3' end position of the Xenopus heat shock gene hsp70A (Bienz, 1984). On transcription pSP65.HS1 gave rise to a 1189 nt precursor mRNA and to 2 other shorter transcripts, again caused by premature transcription termination by the SP6 polymerase.

Transcription products from pSP64.MG1 and pSP65.HS1 were used in matured oocyte microinjection experiments. A parallel injection experiment was carried out using the H4 precursor mRNA as a polyadenylation activity control. Following microinjection RNA samples were taken after 0 and 5 hours incubation of the matured oocytes. Each sample was separated into adenylated and non - adenylated fractions using oligo - dT cellulose, as previously described. Amounts of each fraction, containing RNA from an equal number of oocytes, were then analysed directly by polyacrylamide gel electrophoresis. The result is shown in figure 30. The control experiment using the H4 precursor mRNA confirmed that the polyadenylation activity was present in the matured oocytes and that the oligo - dT binding assay had worked correctly. When the pSP65.HS1 transcription products were injected all 3 of the transcripts became polyadenylated, they bound to oligo - dT cellulose and migrated more slowly on acrylamide gel electrophoresis because of the addition of the poly - A tail. The experiment using pSP64.MG1 RNA gave an interesting result and confirmed a previous observation. It had previously been noticed that the full length pSP64.MG1 transcript did not show a decrease in mobility when injected into embryos, a situation in which the H4 precursor mRNA showed a marked mobility shift (see figure 19). This suggested that the pSP64.MG1 transcript was not a substrate for the polyadenylation activity in embryos. The results of the oligo - dT cellulose binding assay described above have shown that in microinjected matured oocytes

the full length pSP64.MG1 transcript does not become polyadenylated.

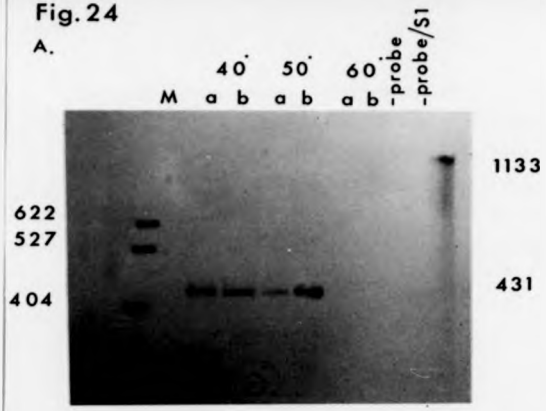
These results suggested that the polyadenylation activity detected in matured oocytes was not a general effect, there was a certain degree of sequence specificity. To confirm this a further experiment was carried out in which a mixture of pSP64.X1H4 and pSP64.MG1 transcription products were coinjected into matured oocytes and the fates of the 2 shorter MG1 transcripts as well as the fates of the full length RNAs were determined. The result of this experiment are shown in figure 31. Immediately after injection all the RNAs were non - polyadenylated and they were all found in the non — polyadenylated fraction, these included the 762 nt full length H4 precursor mRNA, the 444 nt full length MG1 precursor mRNA and the 2 shorter MG1 transcripts. After 5 hours in the oocyte a proportion of the H4 precursor mRNA had been polyadenylated and was found to be located in this fraction. None of the other RNAs became polyadenylated. The 444 nt full length MG1 transcript remained non - adenylated, this band was slightly broader 5 hours after injection because a small amount of the H4 precursor mRNA was 3' cleaved to produce a non - adenylated 446 nt RNA which ran in about the same position as the 444 nt RNA. The shorter MG1 transcripts were unstable in matured oocytes, however the small amount of the 220 nt transcript still remaining after 5 hours was not polyadenylated.

These results have shown that when pSP64.X1H4 and pSP64.MG1 precursor mRNAs were microinjected into the same matured oocytes the H4 RNA became polyadenylated whereas the MG1 transcripts did

not. The polyadenylation activity is specific in that H4, H1 and HS1 transcripts are substrates but MG1 transcripts are not. The basis of this specificity probably does not reside in sequences close to the 3' end of the precursor mRNA as even the prematurely terminated HS1 transcripts became polyadenylated, however the slight possibility that all the HS1 transcripts fortuitously contained the controlling sequence, close to their 3' ends, cannot be formally ruled out.

Fig. 24

A.


40°
50°
60°
M a b a b a b
-probe
-probe/S1
1133
622
527
404
431

B.

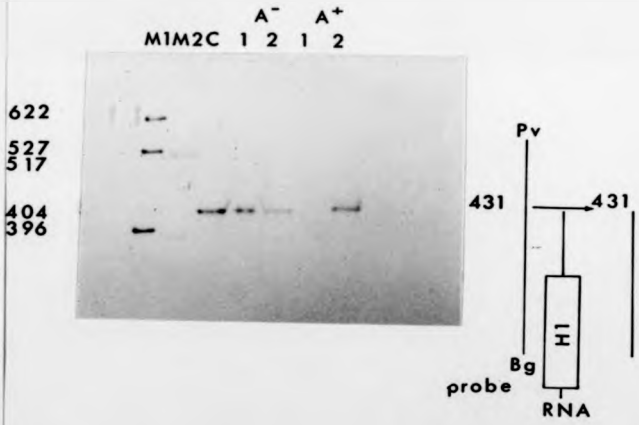

A⁻ A⁺
M1M2C 1 2 1 2
622
527
517
404
396
Pv
431
431
H1
Bg
probe
RNA

Figure 24. S1 nuclease analysis on radioactive precursor mRNA using an unlabelled DNA probe

S1 nuclease analysis was performed on radioactive precursor mRNAs using an unlabelled DNA probe. The fragments of the precursor mRNAs protected by the probe were analysed by acrylamide gel electrophoresis.

Figure 24.A. Optimisation of assay conditions. The probe used in these analyses was a 510 nt Bgl I / Pvu II fragment isolated from pSP64.H1H1. This probe contained 431 nt of sequence upstream of the precursor mRNA 3' end and approximately 80 nt of sequence downstream of the precursor mRNA 3' end. The probe was hybridised to H1 precursor mRNA at 3 hybridisation temperatures. 2 concentrations of S1 nuclease were used for each hybridisation temperature (a = 225 units S1 nuclease / reaction, b = 450 units S1 nuclease / reaction). M = pBR322 Hpa II size marker, - probe = analysis without the addition of the DNA probe, - probe/S1 = analysis without the addition of the probe and without S1 nuclease treatment. 1133 nt = original size of H1 precursor mRNA, 431 nt = size of RNA protected by probe.

Figure 24.B. S1 nuclease analysis on adenylated (A+) and non - adenylated (A-) RNA fractions from matured oocytes injected with H1 precursor mRNA. 1 = RNA from oocytes immediately after injection. 2 = RNA from oocytes 3 hours after injection. M1 = pBR322 Hpa II marker, M2 = pAT153 Hinf I marker, C = Uninjected H1 precursor mRNA. 431 nt = protection by probe to 3' end of H1 precursor mRNA.

Fig. 25

Sequencing of the H4 downstream region in pSP64.XlH4

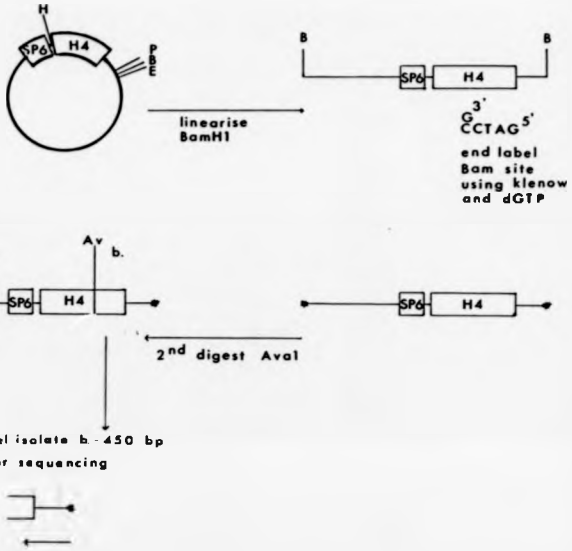


Figure 25. DNA sequencing strategy for the 3' end of the H4 fragment in pSP64.X1H4

pSP64.X1H4 was linearised with Bam HI and the Bam HI ends were radioactively labelled. After labelling the linear pSP64.X1H4 DNA was digested with Ava I. This generated a DNA fragment (b) consisting of SP6 polylinker sequence and about 450 nt of H4 sequence which was isolated and sequenced. The sequence obtained in this way consisted of the SP6 polylinker sequence leading into the sequence of the 3' end of the H4 fragment.

Key to abbreviations of restiction enzyme sites :

H = Hind III P = Pst I B = Bam HI E = Eco RI

Av = Ava I

SP6 = SP6 promoter

Fig. 26

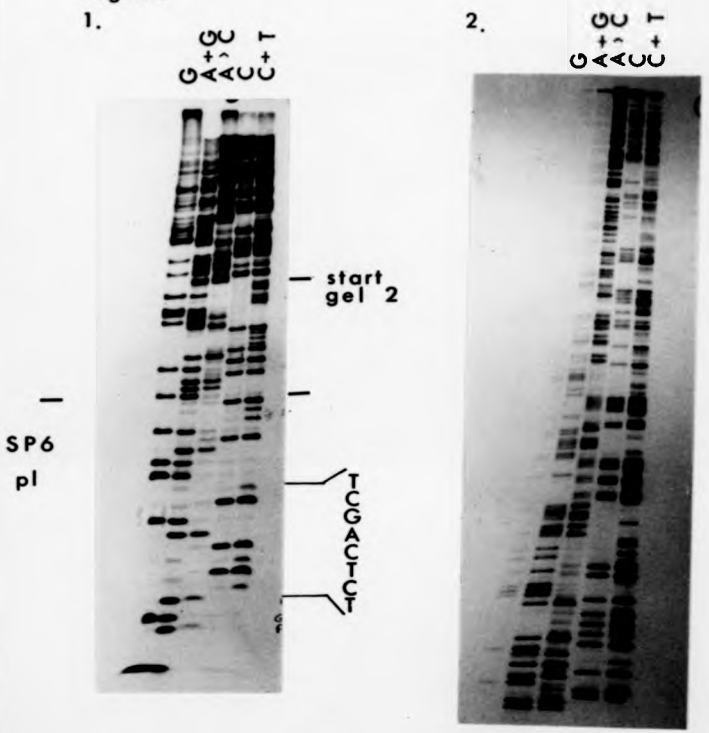


Figure 26. Maxam and Gilbert DNA sequencing of the 3' end of the H4 fragment in pSP64.X1H4

Polyacrylamide gels showing Maxam and Gilbert sequencing of the 3' end of the H4 fragment in pSP64.X1H4.

1. = 20% Acrylamide gel showing the sequence from the labelled restriction site in the SP6 polylinker into the H4 fragment. A sample of sequence from the SP6 polylinker is shown, also marked are the positions where the polylinker sequence ends and the H4 sequence starts and the position where the sequence overlaps the sequence on gel 2.

2. = 8% Acrylamide gel showing more of the sequence of the 3' end of the H4 fragment in pSP64.X1H4.

Fig. 27

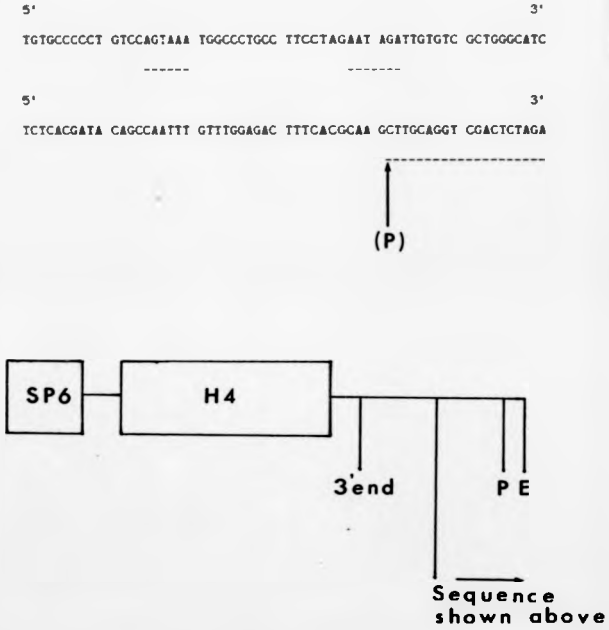

5'
3'
TGTGCCCCCT GTCCAGTAAA TGGCCCTGCC TTCCTAGAAT AGATTGTGTC GCTGGGCATC
5'
3'
TCTCACGATA CAGCCAATTT GTTTGGAGAC TTTCACGCAA GCTTGCAGGT CGACTCTAGA
(P)
SP6
H4
3'end
P E
Sequence
shown above

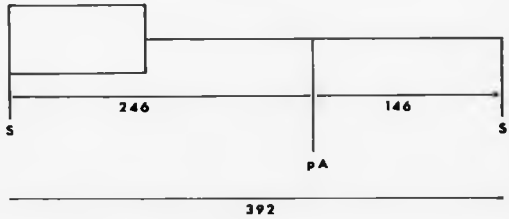
Figure 27. The DNA sequence of the 3' end of the H4 fragment in pSP64.X1H4

The sequence shown represents the 3' 100 nt from the H4 fragment in pSP64.X1H4. The 3' end of this fragment was the Pst I restriction enzyme site (P). This site was altered during the cloning and was not regenerated. The remaining 20 nt of sequence shown is SP6 polylinker sequence (underlined).

2 sequences with 5 out of 6 bases homologous to the AATAAA polyadenylation hexamer are also underlined.

Fig.28

A. 392 bp S fragment from pAW103



B. Cloned in pSP64

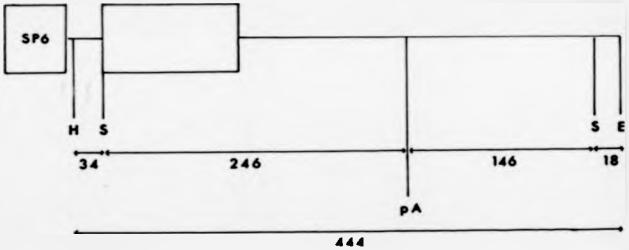


Figure 28. The mouse globin DNA fragment cloned in pSP64.MG1

Figure 28.A The 392 Sau 3A fragment spanning the mouse β globin mRNA 3' end position (pA). This fragment contained 246 nt of sequence from upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of this position.

Figure 28.B The 392 nt Sau 3A fragment cloned into the Bam HI site of pSP64. When linearised with Eco RI and transcribed with SP6 polymerase this sub - clone generated a 444 nt full length precursor mRNA. The precursor mRNA comprised of the globin sequence plus small amounts of SP6 polylinker sequence.

SP6 = SP6 promoter.

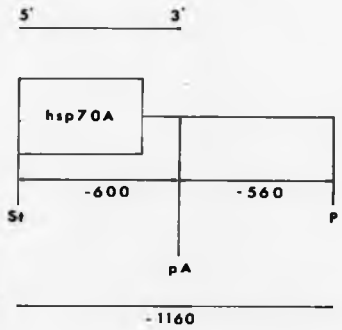
Key to restriction enzyme abbreviations :

H = Hind III S = Sau 3A E = Eco RI

All sizes marked are in nucleotides.

Fig.29

A. The 3' end of the Xenopus hsp70A gene



B. Cloned in pSP65

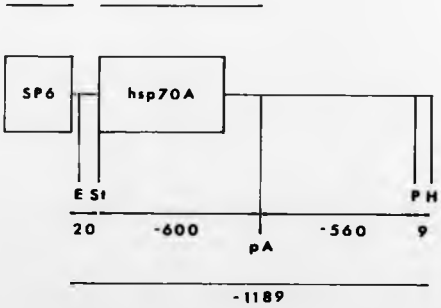


Figure 29. The hsp70A DNA fragment cloned in pSP65.HS1

Figure 29.A. The Sst I / Pst I restriction enzyme fragment spanning the mRNA 3' end position (pA) of the Xenopus hsp70A gene.

Figure 29.B. The 1160 nt Sst I / Pst I hsp70A fragment cloned in the Sst I and Pst I restriction sites of pSP65, this made the sub - clone pSP65.HS1.

Key to abbreviations of restriction enzyme sites :

St = Sst I P = Pst I E = Eco RI H = Hind III

All sizes shown are in nucleotides.

Fig. 30

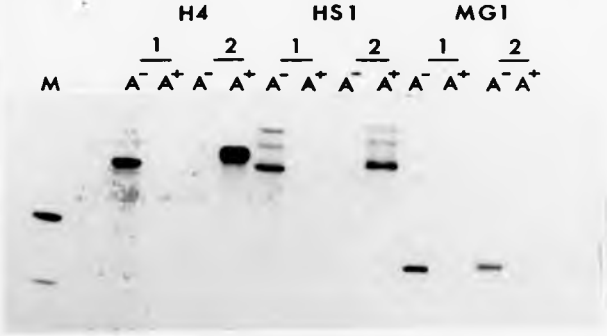


Figure 30. Oligo dT cellulose binding assay of H4, HS1 and MG1 precursor mRNAs after injection into matured oocytes

Radioactively labelled H4, HS1 and MG1 precursor mRNAs were injected into matured Xenopus oocytes. RNA samples were prepared from matured oocytes immediately after injection (1) and from matured oocytes 5 hours after injection (2). These RNA samples were fractionated into adenylated (A+) and non - adenylated (A-) RNA fractions using an oligo dT cellulose batch - binding assay. The RNA fractions were analysed directly by acrylamide gel electrophoresis.

M = pAT153 Hinf I marker.

Fig. 31

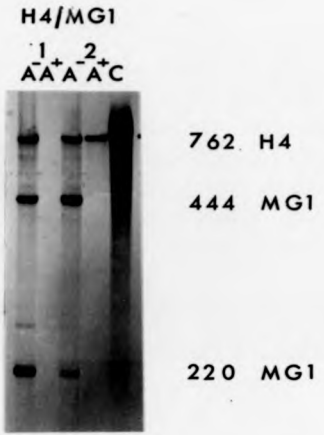


Figure 31. Oligo dT cellulose binding assay on an H4/MG1 precursor mRNA mixture after injection into matured oocytes

Radioactively labelled H4 and MG1 precursor mRNAs were mixed and the mixture injected into matured oocytes. RNA samples were prepared from matured oocytes immediately after injection (1) and from matured oocytes 5 hours after injection (2). The RNA samples were fractionated into adenylated (A+) and non - adenylated (A-) fractions using an oligo dT cellulose batch binding assay. The RNA fractions were analysed directly by acrylamide gel electrophoresis.

1 A- and 1 A+ = Non - adenylated and adenylated fractions from matured oocytes immediately after injection.
2 A- and 2 A+ = Non - adenylated and adenylated fractions from matured oocytes 5 hours after injection.
C = Adenylated RNA fraction from an oligo dT cellulose binding assay using *in vitro* adenylated H4 precursor mRNA.

762 nt = Size of H4 precursor mRNA.
444 nt = Size of MG1 precursor mRNA.
220 nt = Size of prematurely terminated MG1 transcript.

3. The polyadenylation activity and early Xenopus development

H4 precursor mRNA microinjected into matured oocytes and unfertilised eggs becomes polyadenylated, however it was not known whether this was a consequence of microinjection causing activation of the eggs or whether the polyadenylation activity was present in non - activated matured oocytes and eggs. Experiments were carried out to investigate this point. Activation of Xenopus eggs is dependant on free calcium ions (Belanger and Schautz, 1975; Schroeder and Strickland, 1974). Microinjection can be prevented from causing activation by coinjecting EGTA along with the required sample in order to chelate calcium ions. A mixture containing H4 precursor mRNA and 10 mM EGTA was prepared. This mixture was then injected into matured oocytes and early embryos. The embryos were injected as soon as possible after the first cleavage furrow was formed. RNA samples were prepared from immediately after injection and from 5 hours after injection. Parallel experiments were carried out using H4 precursor mRNA without EGTA. RNA samples from matured oocytes and embryos were analysed directly by acrylamide gel electrophoresis. The presence or absence of the polyadenylation activity was judged on whether the H4 precursor mRNA underwent the mobility shift characteristic of polyadenylation.

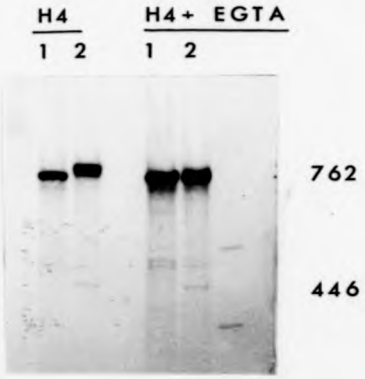
The results of these experiments are shown in figures 32 and 33. In matured oocytes the presence of EGTA was observed to prevent the abortive cleavages associated with activation. EGTA

had no affect on the small amount of 3' cleavage of the histone precursor mRNA that occurs in matured oocytes, however it did inhibit the polyadenylation activity. 2 exposures of the matured oocyte injection experiment autoradiograph are shown, on both, in the absence of EGTA, the H4 precursor mRNA clearly shows a shift in electrophoretic mobility, in the presence of EGTA no mobility shift was detected.

In embryos the H4 precursor mRNA without EGTA underwent both inefficient 3' cleavage and polyadenylation. In the presence of EGTA the same two effects were observed, therefore in embryos EGTA did not inhibit polyadenylation whereas, in matured oocytes it did. The sample from the second time point in the presence of EGTA contains only faint bands, this was a reproducible effect and was assumed to be the result of EGTA having a deleterious effect on the embryo which in turn led to degradation of the H4 precursor mRNA.

Fig. 32

A.



B.

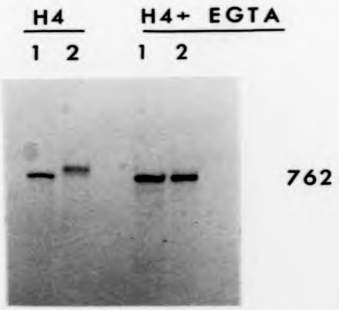


Figure 32. The effect of EGTA on 3' cleavage and polyadenylation of H4 precursor mRNA in matured oocytes.

Radioactively labelled H4 precursor mRNA was coinjected with 10 mM EGTA into matured Xenopus oocytes. RNA samples were prepared from matured oocytes immediately after injection (1) and from matured oocytes 5 hours after injection (2). These RNA samples were analysed directly by acrylamide gel electrophoresis.

Figure 1.A. H4 = RNA samples from matured oocytes injected with H4 precursor mRNA.
H4 + EGTA = RNA samples from matured oocytes injected with H4 precursor mRNA + 10 mM EGTA.
762 nt = Size of H4 precursor mRNA.
446 nt = Size of 3' cleaved H4 RNA.

Figure 1.B. Shorter exposure of autoradiograph shown in figure 1.A.

Fig.33

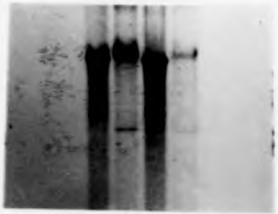


Figure 33. The effect of EGTA on 3' cleavage and polyadenylation of H4 precursor mRNA in embryos

Radioactively labelled H4 precursor mRNA was coinjected with 10 mM EGTA into Xenopus embryos at the 2 cell stage. RNA samples were prepared from embryos immediately after injection (1) and from embryos 5 hours after injection (2). The RNA samples were analysed directly by acrylamide gel electrophoresis.

H4 1 and H4 2 = RNA samples from embryos injected with H4 precursor mRNA.
H4 + EGTA 1 and H4 + EGTA 2 = RNA samples from embryos injected with H4 precursor mRNA + 10 mM EGTA.

762 nt = Size of H4 precursor mRNA.
446 nt = Size of 3' cleaved H4 RNA.

c. Discussion

The results presented in the first part of this chapter demonstrated that, to within 5 - 10 nt, the 3' end of H1 precursor mRNA polyadenylated in the matured oocyte was in the same position as the 3' end of non - adenylated H1 precursor mRNA. This result suggested that polyadenylation of the H1 precursor mRNA was not linked to a 3' cleavage reaction, unless the reaction removed only a very small amount of sequence from the 3' end of the precursor mRNA. This left 2 possibilities as to the site of action of the activity : either it added a poly - A tail directly to the 5' end of the precursor mRNA or it added a poly - A tail directly to the 3' end of the precursor mRNA. The 5' end of the RNA was capped prior to injection and any activity that added a poly - A tail to a capped RNA 5' end would be very unusual, this possibility was considered unlikely. It was therefore concluded that the activity added the A tail directly to the 3' end of the precursor mRNA. Based on the fact that there was no 3' cleavage of the precursor prior to polyadenylation the length of the A tail added was calculated. The shift in mobility of the H1 precursor mRNA on acrylamide gel electrophoresis was a reflection of the length of the A tail added. This mobility shift was measured and used to calculate the A tail length. For the H1 precursor mRNA the average length of A tail added was about 60 - 70 A residues. This A tail length is shorter than the average length of A tails on cellular mRNAs which is about 200 - 300 A residues, however perhaps more significantly the average A tail length on the prominent population of transcripts in the Xenopus

oocyte is about 60 A residues (Cabada et al, 1977). As already pointed out the A tail added to microinjected H1 precursor mRNA is the result of a controlled polyadenylation activity which adds a relatively constant number of A residues to each RNA molecule. This is in contrast to purified poly - A polymerase enzymes from a number of sources which add A tails of varying lengths when used *in vitro* (for review see Edmonds, 1982).

Two explanations for the apparent control of the polyadenylation activity were considered. It was thought possible that a specific sequence within the precursor mRNAs could be involved both in causing polyadenylation to occur and in determining the length of A tract added. Alternatively it was thought possible that the polyadenylation activity was a general effect reflecting a change in the overall balance between polyadenylation and poly - A degradation in favour of poly - A synthesis, in this case the acivity was expected to use any injected precursor mRNA as a substrate. I will discuss each of these possibilities in turn.

The best candidate for a sequence controlling the polyadenylation activity was the hexamer AAUAAA. This hexamer has been shown to be necessary for the 3' cleavage of precursor mRNAs which usually preceeds polyadenylation in the nucleus (see introduction). In addition considerable evidence has now accumulated that the hexamer can also direct polyadenylation in the absence of 3' cleavage. A HeLa cell extract was used to investigate the polyadenylation reaction *in vitro* (Manley, 1983), in this system it was shown that when SV40 precursor mRNAs

were added they acted as substrates for a controlled polyadenylation activity. This activity was independant of any cleavage reaction, it simply added an A tail to the existing end of the precursor mRNA. Not all the precursor mRNAs added to the system were polyadenylated, the activity was found to be far more efficient when the 3' end of the precursor mRNA was close (within 45 nt) to the natural mRNA 3' end position. This result suggested that sequences close to the mRNA 3' end position were important in the polyadenylation reaction, these could possibly include the AAUAAA hexamer. A similar HeLa cell extract was shown to polyadenylate the 3' ends of added adenovirus precursor mRNAs (Moore and Sharp, 1984). These precursors, which contained more than 1kb of sequence past the mRNA 3' end position, were only polyadenylated at low efficiency, about 12% of the added precursor mRNA was polyadenylated directly at it's existing 3' end.

Further work with this HeLa cell extract directly implicated the AAUAAA hexamer in polyadenylation in the absence of 3' cleavage (Manley et al, 1985). These results demonstrated that when a fragment containing sequences around the SV40 early mRNA 3' end position was added to precursor mRNAs, previously shown not to be efficiently polyadenylated in the extract, the ability to be polyadenylated was conferred. Only precursor mRNAs containing the AAUAAA hexamer were substrates for the polyadenylation activity. It was also shown that the AAUAAA hexamer could direct the polyadenylation of a precursor mRNA 3' end more than 400 nt further downstream, although this was less

efficient than when the hexamer was close to the end of the precursor mRNA.

More recent work (Zarkower et al, 1986) described the use of a HeLa cell nuclear extract to investigate the polyadenylation of SV40 late transcripts *in vitro*. In these experiments artificial RNAs were synthesised in which the RNA 3' end was in the normal mRNA 3' end position, 12 nt downstream from a AAUAAA hexamer. When these precursor mRNAs were added to the system they were efficiently polyadenylated at the mRNA 3' end position. Mutant precursor mRNAs in which the only change was the conversion of the AAUAAA sequence to a AACAAA sequence were not polyadenylated in the extract. These results suggested that the AAUAAA hexamer was involved in the polyadenylation reaction and that this exact sequence was required for efficient polyadenylation.

Based on the evidence that the AAUAAA hexamer can direct polyadenylation of mRNA 3' ends in the absence of 3' cleavage the sequences close to the 3' ends of the H1 and H4 precursor mRNAs were examined to see if they contained copies of the hexamer. Both precursor mRNAs have 36 nt of SP6 polylinker sequence directly at the 3' end, this was known not to contain the AAUAAA sequence. The H1 precursor mRNA was found to contain the AAUAAA hexamer 60 nt upstream from the precursor mRNA 3' end (see figure 23). However when the H4 gene was partially sequenced it was found not to contain a copy of the hexamer within 140 nt of the precursor mRNA 3' end (see figure 24). Sequences with some similarity to the hexamer were however found,

these included the sequence AAUAGA about 95 nt upstream from the precursor mRNA 3' end and the sequence AGTAAA about 115 nt upstream from the precursor mRNA 3' end. The amount of the H4 sequence obtained and examined was based on two facts : firstly that most genes contain a copy of the AAUAAA sequence within 10 - 30 nt of the mRNA 3' end position which suggested that the hexamer might need to be close to a 3' end to direct polyadenylation and secondly the finding that *in vitro* only precursor mRNAs with 3' ends close to the normal mRNA 3' end position were polyadenylated efficiently (Manley, 1983). In the light of more recent evidence, that a 3' end as far as 400 nt downstream from an AAUAAA sequence can be polyadenylated, it might have been better to obtain more of the H4 sequence (Manley et al, 1985). Whether either of the 2 sequences similar to the AAUAAA hexamer found in the H4 precursor mRNA was responsible for directing polyadenylation remains an open question. *In vitro* it has been shown that an AACAAA sequence will not substitute for the AAUAAA sequence in directing polyadenylation of a downstream RNA 3' end (Zarkower et al, 1986), however the efficiency of other similar sequences has not been tested. In nature the sequence AGTAAA does occur upstream of an mRNA 3' end position where it presumably functions in directing 3' cleavage and polyadenylation, whether it could direct polyadenylation in the absence of 3' cleavage is not known (Birnstiel et al, 1985).

The experiments in which the pSP65.HS1 and pSP64.HG1 transcripts were injected gave further evidence relevant to the way in which the polyadenylation activity might be controlled.

These experiments demonstrated that 3 HS1 transcripts, one full length and two prematurely terminated, were polyadenylated after microinjection into matured oocytes. In similar circumstances neither the full length or prematurely terminated MG1 transcripts were polyadenylated. The coinjection experiment in which the MG1 transcripts were injected into the same oocytes as the H4 precursor mRNA demonstrated that the lack of poladenylation of the MG1 transcripts was not simply due to an inhibitor in the MG1 RNA preparation. The fact that all 3 of the HS1 transcripts became polyadenylated suggested that it was unlikely that their polyadenylation was under the control of the AAUAAA sequence. The smallest HS1 transcript was about 427 nt smaller than the largest, if one AAUAAA sequence common to all the transcripts was responsible for controlling the polyadenylation activity it would need to have exerted its effect over a long distance (at least 427 nt) to have caused the polyadenylation of the longest transcript. Alternatively it could be argued that each of the transcripts contained a copy of the AAUAAA sequence close to its 3' end. The sequence of the downstream region of this gene is not available but it would be suprising if each transcript had a copy of the AAUAAA hexamer close to its 3' end.

In addition the full length pSP64.MG1 transcript was known to contain 2 copies of the AAUAAA hexamer, the nearest within 75 nt of the 3' end of the precursor mRNA. If this hexamer was responsible for controlling the polyadenylation of the histone and HS1 precursor mRNAs why was the full length MG1 transcript not polyadenylated ?

From the available evidence it remains possible that polyadenylation of the histone precursor mRNAs was controlled by the AAUAAA sequence in the case of the H1 precursor mRNA and by either one of the sequences similar to AAUAAA already identified in the H4 precursor mRNA or by an AAUAAA sequence in a region of the H4 precursor mRNA not sequenced. It is unlikely that polyadenylation of all 3 of the HS1 transcripts could be controlled by either a single AAUAAA sequence common to all the transcripts or by AAUAAA sequences located by coincidence close to the 3' end of each of the RNAs. In the first case it would be expected that the shortest transcript with the AAUAAA nearest to its 3' end would be polyadenylated more efficiently than the longest transcript with the AAUAAA a long way from its 3' end. It was however observed that all 3 of the HS1 transcripts were polyadenylated equally efficiently. The activity that polyadenylates the HS1 transcripts is likely to be the same activity and to work in the same way as the activity that polyadenylates the histone transcripts. If the HS1 transcripts are polyadenylated in a reaction independent of an AAUAAA sequence then the histone transcripts probably are also.

The possibilty remained that the polyadenylation activity was controlled by a previously unidentified sequence common to the H4, H1 and HS1 precursor mRNAs but absent from the MG1 RNAs. If this sequence did exist it would need to be equally efficient at causing polyadenylation of mRNA 3' ends located far downstream and those located close by. To attempt to identify this sequence it would be necessary to obtain the downstream

sequence of the heat shock gene in the HS1 precursor mRNA and compare it with the H4 and H1 precursor mRNA sequences, inject mutant precursors with sequences deleted in an attempt to abolish polyadenylation and test a number of other precursor mRNAs for polyadenylation under identical conditions.

It was considered more likely that the polyadenylation activity was a general activity with no specific sequence requirements. In this case the length of the A tail would be controlled by the balance between polyadenylation and poly - A degradation. A comparable increase in polyadenylation has been shown to occur in sea urchin early development. Between fertilisation and the two cell stage in the sea urchin the poly - A content of the embryo doubles (Slater et al, 1973). This increase is mainly the result of the addition of A tails to RNAs made during oogenesis (Slater and Slater, 1974). Some of these RNAs are non - adenylated prior to fertilisation and become adenylated whilst other have short A tracts which increase in length. The activity, which can occur in the absence of a nucleus , probably results from the A tails being in a dynamic state and the adenylation activity being stronger than the de - adenylation activity (Wilt, 1977). If a general polyadenylation activity is the correct explanation for the activity detected in microinjected matured oocytes the lack of polyadenylation of the pSP64.MG1 transcripts needs to be explained. These transcripts may not have acted as substrates for the polyadenylation activity because they assumed a secondary structure in which the 3' end was inaccessible to the polyadenylation activity or because they

contained a sequence which inhibited the polyadenylation activity.

The significance of the polyadenylation activity in Xenopus development is very difficult to assess. It has been shown that microinjected H4 and H1 precursor mRNAs, which are substrates for the activity in injected matured oocytes, are not polyadenylated in the nucleus or cytoplasm of stage 6 oocytes. The experiments in which the H4 precursor mRNA was coinjected with EGTA into matured oocytes were designed to provide information on which stage in development the activity was first detectable. On injection matured oocytes and unfertilised eggs are activated, a process requiring calcium ions. It was felt that the polyadenylation activity may have been a result of activation and would not be present in non - activated matured oocytes. By injecting the H4 precursor mRNA and EGTA into matured oocytes this was investigated. It was found that in matured oocytes EGTA inhibited the polyadenylation activity but did not inhibit the inefficient 3' cleavage of the H4 precursor mRNA. Therefore in this system the 3' cleavage reaction did not require calcium ions. Two explanations for the inhibition of the polyadenylation activity were considered : either the activity only appeared after activation of the matured oocyte, when activation was inhibited the polyadenylation activity was not detectable, or calcium ions were required as an essential cofactor for the poly -A polymerase enzyme responsible for the polyadenylation activity.

In an attempt to resolve this, the same experiment was performed using embryos instead of matured oocytes. It had been shown that embryos did polyadenylate microinjected H4 precursor mRNA. It was thought that any polyadenylation activity dependant upon activation of the egg would be present in an early cleavage embryo whether calcium was present or not. Alternatively if the polyadenylation activity required calcium ions as a cofactor removing these ions from the embryo would have inhibited the activity just as it did in matured oocytes. It was found that when the H4 precursor mRNA was coinjected with EGTA into early embryos 3' cleavage and polyadenylation were both unaffected. The simplest interpretation of these results was that the polyadenylation activity detected in microinjected matured oocytes was a consequence of microinjection activating the oocytes, the polyadenylation activity did not require calcium ions as a cofactor.

Work has been carried out using *in vitro* systems to investigate the cofactor requirements for the cleavage and polyadenylation of precursor mRNAs. A HeLa cell nuclear extract was shown to cleave and polyadenylate added Adenovirus precursor mRNAs. In the presence of EDTA, which chelates both calcium and magnesium ions, the 3' cleavage reaction proceeded normally however the polyadenylation reaction was inhibited and produced shorter than normal A tails (Moore and Sharp, 1985). A similar HeLa cell nuclear extract has been shown to 3' cleave added mouse histone H4 precursor mRNAs (Gick et al, 1986). In the presence of EDTA this 3' cleavage reaction proceeded normally. The 3'

cleavage and polyadenylation of SV40 late precursor mRNAs in the HeLa cell nuclear extract have also been studied (Zarkower et al, 1986). In the presence of EDTA 3' cleavage was unaffected but polyadenylation was completely inhibited. These *in vitro* results suggest that 3' cleavage is independent of calcium and magnesium ions. The results from the injection of the H4 precursor mRNA and EGTA into matured oocytes were consistant with the *in vitro* results, removing calcium ions did not inhibit 3' cleavage in matured oocytes.The *in vitro* results also suggested that removing calcium and magnesium ions inhibits the polyadenylation activity linked to 3' cleavage. Bacterial poly - A polymerases require magnesium ions but not calcium ions for their activity (for review see Edmonds, 1982). This suggests that the probable explanation for the loss of polyadenylation activity in the *in vitro* studies was the removal of magnesium ions rather that the removal of calcium ions.If this explanation is correct it supports the conclusion that in the experiments where H4 precursor mRNA and EGTA were coinjected into matured oocytes the prevention of activation rather than the removal of a cofactor was responsible for the lack of a detectable polyadenylation activity.

It was concluded that the polyadenylation activity detected in microinjected matured oocytes was probably a reflection of a change due to activation rather than being a genuine matured oocyte activity, however because of the difficulties in interpreting the results of these experiments the alternative was also considered.

In Xenopus changes in polyadenylation on maturation have been shown to occur. The initial response of Xenopus eggs to treatment with progesterone is a 5 - 10% increase in adenylated sequences and an increase in the average poly - A tail length of 10 - 20 nt. After the germinal vesicle breaks down 30 - 35 % of the total adenylated RNA disappears (Darborough and Ford, 1979). Changes in the polyadenylation status of known sequences also occur, for example histone mRNAs are deadenylated on oocyte maturation (Ballantine and Woodland, 1985).

However if the conclusion from the H4 precursor mRNA and EGTA injection experiments are correct the polyadenylation activity detected in matured oocyte, unfertilised egg and embryo injections is not a reflection of polyadenylation changes at maturation, instead the activity represents a change occuring at activation / fertilisation. This makes it analogous to the change observed in sea urchin embryos after fertilisation, where the poly A content of the embryo doubles between fertilisation and the 2 cell stage (Slater et al, 1973). Other work (Sagata et al, 1980) has shown that a similar effect does occur in Xenopus. After fertilisation or activation of Xenopus eggs there is a steady increase in the amount of adenylated RNA in the embryo until the blastula stage. This increase involves the adenylation of existing maternal RNAs because there is no new transcription in the embryo until the MBT. The results obtained from the precursor mRNA injection experiments probably reflect this increase.

3.5 3' END FORMATION OF MOUSE β GLOBIN mRNA IN THE OOCYTE NUCLEUS - DNA INJECTION EXPERIMENTS

a. Introduction

The aim of these experiments was to establish an experimental system to investigate the mechanisms used to form the 3' end of a polyadenylated mRNA. This system was based on injecting cloned genes into the Xenopus oocyte nucleus and analysing the 3' end formation of the transcribed mRNA. This approach has been used sucessfully to identify the sequences, and a factor, involved in the 3' processing of sea urchin histone mRNAs (Birchmier et al, 1982; 1983; 1984; Stunnenberg and Birnstiel, 1982; Galli et al, 1983). Histone mRNAs are however unlike the majority of eukaryotic mRNAs, they are normally not polyadenylated, their genes lack the conserved sequences found around polyadenylated mRNA 3' end positions and they have other conserved 3' sequences including a 3' terminal stem and loop structure (see Birchmier et al,1983). The success of the oocyte microinjection technique at elucidating the 3' processing mechanisms for histone mRNAs made it logical to use a similar approach to investigate the 3' processing of a typical polyadenylated mRNA.

The gene used in these experiments was the mouse β (major) globin gene. This gene has been completely sequenced (Konkel et al, 1978). It has also been used extensively in studies of transcription termination. In isolated nuclei from mouse EL cells

it has been shown that transcription terminates more than 700 nt downstream from the mRNA 3' end position (Citron et al, 1984), this generates a precursor mRNA with a 3' extension which undergoes RNA processing to generate the mature mRNA. This processing involves the removal of the 3' extension to a point 19 nt downstream from the AAUAAA hexamer and immediately downstream from the CAYUG sequence. The globin mRNA 3' end formed in this reaction is polyadenylated. A second conserved sequence block associated with polyadenylated mRNA 3' end positions , rich in G and T residues, is found downstream of the mRNA 3' end position in the mouse β globin gene. 94 nt downstream from the site where the mRNA 3' end is formed is a second AATAAA hexamer followed by the sequence CACTT 7 nt further downstream. This second potential 3' end site is not used at a detectable level in mouse erythrocytes (see figure 35). The mouse β globin gene was used as part of a hybrid gene called pAV103 (Wilson, pers. comm.). pAV103 consisted of the 5' end of a Xenopus H3 gene fused to the 3' end and downstream sequence from the mouse β globin gene. pAV103 was preferred over the whole globin gene for oocyte injections because the presence of a Xenopus histone promoter ensured a high level of expression. pAV103 contained only the 5' end of the histone so that the only 3' end determining sequences present were those of the globin gene. In effect investigating 3' end formation of pAV103 transcripts was the same as investigating 3' end formation of globin transcripts.

On injection of pAV103 DNA into the oocyte nucleus 2 general results were considered: either the pAV103 transcripts would have

their 3' ends at the mouse globin mRNA 3' end position and these ends would be polyadenylated, or the pAW103 transcripts would have 3' ends at positions other than the mouse globin mRNA 3' end position and / or these 3' ends would not be polyadenylated. In the first case it was then planned to make deletions in sequences close to the globin mRNA 3' end position to investigate the sequences necessary for 3' end formation. In the second case it was planned to add back factors, initially fractions from mouse cells containing snRNAs, to the system in an attempt to complement the lesion in the 3' processing mechanism. Initial experiments were performed to determine whether histin transcripts made in the oocyte from injected DNA had their 3' ends at the mouse globin mRNA 3' end position and to determine if these ends were polyadenylated.

b. Results

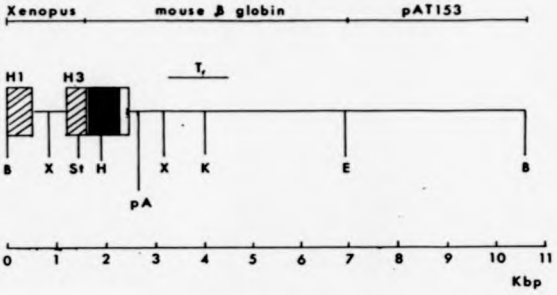
Injection of pAW103 DNA into the oocyte nucleus was performed as described in materials and methods. In a typical experiment 20 oocytes were injected with the DNA. The injected oocytes were incubated overnight to allow an easily detectable level of pAW103 mRNA to accumulate. The oocytes were then homogenised and total RNA was prepared from them. A similar number of uninjected oocytes were also homogenised to produce a control RNA sample. RNAs from pAW103 injected and uninjected control oocytes were then used in a S1 nuclease analysis to map the 3' ends of the pAW103 transcripts. The probe used was a 3'

end labelled Sau 3A fragment that spanned the ß globin mRNA 3' end position (see figure 34). The full length of this probe was 392 nt. Transcripts with the 3' end at the globin mRNA 3' end position protected 246 nt of the probe from S1 nuclease digestion. pAW103 transcripts with 3' ends at aberrant positions would have protected different lengths of the probe. The results of this S1 nuclease analysis are shown in figure 35. The probe was also used, under identical conditions, on RNA obtained from mouse red blood cells. This RNA contained mouse ß globin mRNAs with genuine 3' termini. The result of this analysis, also shown in figure 35, allowed the globin mRNA 3' end produced on pAW103 transcripts in the oocyte to be compared with the 3' end of genuine mouse ß globin mRNA. The strong band at 246 nt in the mouse globin RNA track was caused by protection of the S1 probe to the 3' end of authentic globin mRNA. This strong band is also in the 4 tracks that contained RNA samples from pAW103 injected oocytes, in these tracks the band was caused by pAW103 transcripts ending at the globin mRNA 3' end position. As expected the 246 band was completely absent from the uninjected control oocyte RNA track where no RNA complementary to the probe was present. There are faint bands shorter than the 246 band in the pAW103 injected samples. These might have been caused by low abundance pAW103 transcripts with 3' ends at abnormal positions. It is more likely that they were caused by weak base pairing between the pAW103 mRNA and the probe at certain sequences allowing the S1 nuclease to cut. The bands shorter than the 246 band in the mouse globin RNA track were probably the result of a

number of factors. In addition to S1 artefacts caused by weak base pairing between probe and mRNA, shorter bands also arose from mismatching of the probe with transcripts from the other mouse globin genes and from degradation of the mouse globin mRNA. The last point was a significant factor. The globin RNA was stored at - 20° C in S1 hybridisation buffer. It was very noticeable that the number of these shorter bands increased markedly the longer the RNA was stored. The strong band at 392 nt, in the pAV103 injected and control RNA tracks, was the size of the full length probe. Its presence in the control RNA track, where no globin mRNA was present, indicates that the same band in the pAV103 RNA track was mainly caused by rehybridisation of the probe, rather than by full length protection of the probe by pAV103 transcripts. Whether the 392 band is seen or not depends on the ratio of probe to its complementary RNA. In the mouse globin RNA analysis the 392 band is absent because the probe was not in such a large excess over its complementary RNA. It is possible that the pAV103 injected RNA sample did contain a small number of transcripts with 3' ends further downstream than the end of the probe and that these contributed, to a small extent, to the intensity of the 392 band by causing full - length protection of the probe. There are no bands visible in the pAV103 injected RNA tracks, between the 246 and 392 bands, this means that there were no transcripts present with 3' ends made between the globin mRNA 3' end position and the end of the probe, 146 nt further downstream.

Fig. 34

A. pAW103



B. The 3' end of the mouse β globin gene

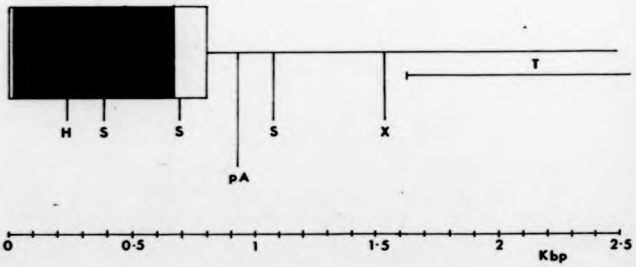


Figure 34. Restriction enzyme map of pAW103

Figure 34.A The entire pAW103 clone (Wilson, pers. comm.) with the Xenopus histone sequences, the mouse globin sequences and the pAT153 vector sequences marked.

Figure 34.B. A portion of the mouse globin gene, from pAW103, including the globin mRNA 3' end position.

The blacked out portion of the globin gene represents an intron sequence.

T = Transcription termination region (Citron et al, 1984).

pA = Site of polyadenylated 3' terminus of mouse globin mRNA.

Key to abbreviations of restriction enzyme sites :

B = Bam HI X = Xba I St = Sst I H = Hind III

K = Kpn I E = Eco RI S = Sau 3A

Fig. 35

pAW103

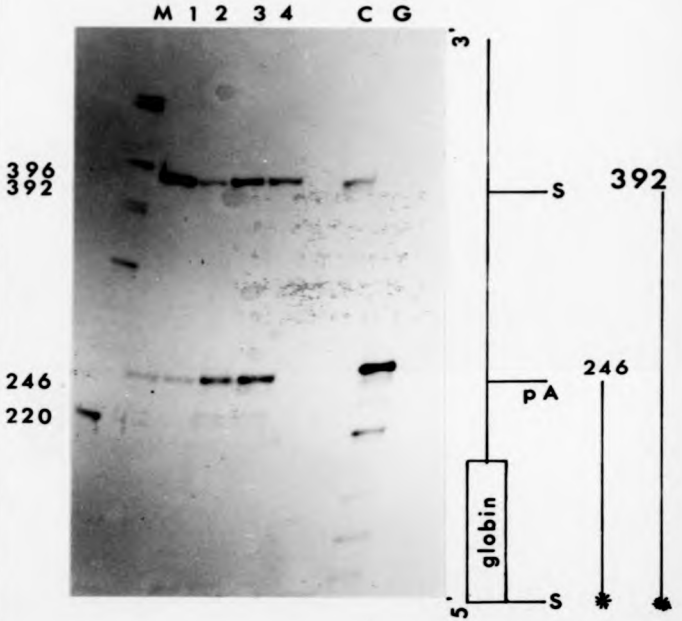


Figure 35. S1 nuclease mapping of the 3' ends of pAW103 transcripts made in the oocyte nucleus

pAW103 DNA was injected into the nucleus of Xenopus oocytes. The injected oocytes were incubated overnight and total RNA was prepared from them. RNA from injected oocytes was analysed in a S1 nuclease assay using a 3' end - labelled DNA probe that spanned the globin mRNA 3' end position in pAW103. The fragments of the probe protected from S1 nuclease digestion were analysed by electrophoresis on a polyacrylamide gel.

The DNA probe used was a 392 nt Sau 3A fragment that contained 246 nt of sequence from upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of this position.

M = pBR322 Hinf I marker

pAW103 1 and pAW103 2 = S1 nuclease analysis on duplicate RNA samples from oocytes injected with pAW103 DNA and incubated overnight, each S1 analysis contained 1 oocyte equivalent of RNA (5 ug).

pAW103 3 and pAW103 4 = Identical S1 analyses to samples 1 and 2 except that each S1 analysis contained 2 oocyte equivalents of RNA (10 ug).

C = Control S1 nuclease analysis using RNA from uninjected oocytes.

G = S1 nuclease analysis on authentic mouse globin mRNA.

392 nt = Full length of Sau 3A probe, 246 nt = Protection of probe to globin mRNA 3' end position (pA).

Further experiments were then performed to determine if the pAW103 transcripts made in the oocyte were polyadenylated.

pAW103 DNA was injected into the nucleus of about 20 oocytes. After overnight incubation of the oocytes, total oocyte RNA was prepared, this oocyte RNA contained pAW103 transcripts. The total RNA was fractionated into adenylated and non - adenylated fractions using an oligo - dT cellulose batch binding assay. A S1 nuclease assay was then used to determine which fraction contained the pAW103 transcripts. In the S1 nuclease assay, the same 392 nt end - labelled Sau 3A fragment, as had been used previously, was used. Each analysis contained an amount of either adenylated or non - adenylated RNA from an equal number of injected oocytes. After S1 nuclease treatment the S1 protected products were analysed by polyacrylamide gel electrophoresis followed by autoradiography. The results of this experiment are shown in figure 36. The 246 nt band, representing pAW103 transcripts with 3' ends at the globin mRNA 3' end position, was located exclusively in the polyadenylated RNA fraction, this indicated that pAW103 transcripts made in the oocyte nucleus were polyadenylated. There was no trace of this band in the non - adenylated fraction, therefore all the transcripts with their 3' end at the globin mRNA 3' end position were polyadenylated. Both adenylated and non - adenylated fractions contained a strong band at 392 nt. This 392 band was mainly caused by renaturing of the probe with possibly some contribution, in the case of the non - adenylated fraction, fromhybridisation of the probe to injected pAW103 DNA. The band shorter than the 392 nt band, labelled Cf,

Fig. 36

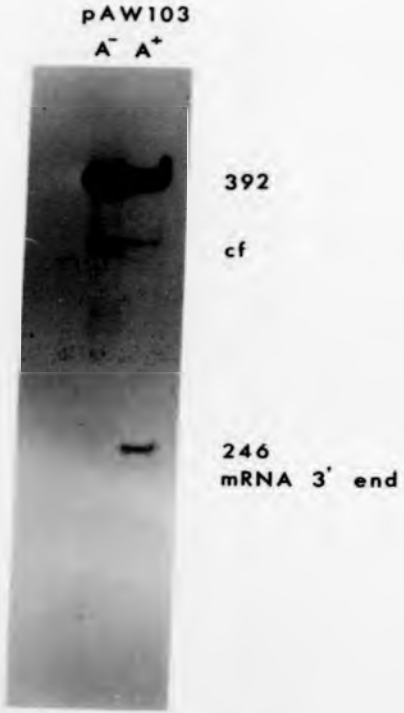


Figure 36. S1 nuclease analysis on adenylated and non - adenylated RNA fractions from oocytes injected with pAW103 DNA

Oocytes were injected with pAW103 DNA and after overnight incubation total RNA was prepared. The total RNA was fractionated into adenylated (A+) and non - adenylated (A-) fractions using an oligo dT cellulose batch binding assay. RNA samples from each fraction were subject to S1 nuclease analysis using the 392 nt 3' end - labelled Sau 3A globin probe.

pAW103 A- and pAW103 A+ = S1 nuclease analyses on fractionated RNAs from oocytes injected with pAW103 DNA.

392 nt = Size of full length S1 probe.

cf = Contaminating fragment in probe preparation.

246 nt = Protection of probe to globin mRNA 3' end position in pAW103.

arose as a consequence of the way in which the probe was prepared for these experiments. The probe was made by gel isolating the required 392 nt Sau 3A fragment from a total Sau 3A digest of pSP64.MG1. A small amount of a contaminating Sau 3A fragment, very similar in size to the required fragment, was present in the DNA used to make the probe. The contaminating fragment was labelled and a certain amount of it escaped digestion in the S1 analysis. The band labelled cf was detected as a result. The contaminating Sau 3A fragment had no affect on the S1 nuclease analysis because it contained only vector sequence from the pSP64 plasmid.

c. Discussion

The experiments presented in this section have demonstrated that on injection of pAV103 DNA into the oocyte nucleus, transcripts with their 3' ends at the mouse globin mRNA 3' end position were produced. All the transcripts with the 3' end at the globin mRNA 3' end position were polyadenylated. These results indicate that the Xenopus oocyte contains mechanisms that recognise and interact with the sequences that control mouse globin mRNA 3' end formation. The fact that the oocyte possesses these mechanisms was not suprising. The oocyte requires a mechanism to form the 3' ends of its own polyadenylated transcripts. From the conservation of sequences around mRNA 3' end positions, between different genes and different species, it would appear that there is a common basic mechanism for making the 3' ends of polyadenylated mRNAs. The mechanism in the oocyte

is sufficiently similar to the mechanism used in the mouse, for globin mRNAs, that the globin mRNA 3' end position can be recognised in the oocyte. In mouse erythroleukemia cells the β globin gene is transcribed past the mRNA 3' end position (Citron et al, 1984). A precursor mRNA is generated which must undergo 3' processing to produce the mature globin mRNA. The sequences around the mouse globin mRNA 3' end position control this mechanism for making mRNA 3' ends by cleavage of a longer precursor mRNA. Although there is no direct evidence that transcription of pAW103, downstream of the mRNA 3' end position does occur in oocytes, it would be very unlikely that a different mechanism to form the globin mRNA 3' end would be used. The pAW103 gene contains only the 3' end of the mouse globin gene, the 5' end of pAW103 is provided by a Xenopus histone H3 gene. The fact that pAW103 mRNA was made with the 3' end at the globin mRNA 3' end position confirms that sequences at the 5' end of the mRNA are not necessary for accurate 3' end formation. In this case a histone 5' end did not affect globin mRNA 3' end formation.

The experiments presented in this chapter also demonstrated that all the pAW103 transcripts with the 3' end at the globin 3' end position were polyadenylated. This might have been because 3' cleavage and polyadenylation were linked in some way, or because there was a very efficient polyadenylation activity that worked independantly of 3' cleavage. No transcripts were detected with 3' ends formed at sites in the globin gene between the usual globin mRNA 3' end position and the end of the S1 probe, 146 nt

further downstream. mRNA 3' ends were not made at the second possible mRNA 3' end position which lies in this region. A comparison between the sequences around the usual mRNA 3' end position ,which was used in the oocyte ,and the second potential mRNA 3' end position which was not used, is shown in figure 37.

Figure 37. The DNA sequence of the mRNA 3' end position of the mouse ß major globin gene in pAW103

(sequence - Konkel et al, 1978; underlined AATAAA hexamers and CATTG sequences ; arrows indicate positions of potential mRNA 3' ends, ***** indicates position of the 5 base pair complement to the AATAAA sequence)

Both sites contain the AATAAA hexamer and a match to the CATTG sequence (although not a perfect match in site 2) and both might be expected to be utilised. Presumably sequences downstream of the potential mRNA 3' end positions determine, that even in a heterologous system, the second site is not used.

In the results section the possibility that transcripts past the 3' end of the S1 probe were present in the injected oocytes was discussed. It was concluded that in the steady state RNA population these long transcripts were not very abundant. Such long transcripts might have been genuine precursor mRNAs produced as a result of transcription termination downstream of the mRNA 3' end position. Because these were not detected it suggests that either they were rapidly 3' cleaved to the mature mRNA size or that 3' cleavage was inefficient but that unprocessed precursor transcripts were unstable and were rapidly degraded. It would be necessary to compare the transcription rate of pAW103 DNA with the amount of mature pAW103 mRNA produced to resolve this issue. Long transcripts might also have resulted from mRNA 3' processing to sites downstream of the normal mRNA 3' end site. Transcripts with 3' ends made in this way could be polyadenylated and stable. These transcripts were not detected so this aberrant processing does not happen to any great extent.

Various genes have been microinjected into oocytes and the polyadenylation status of their transcripts determined. Injection of SV40 DNA and analysis of transcripts from the SV40 late region demonstrated that in the oocyte these transcripts had the 3' end in the correct position and they were predominantly

polyadenylated (Wickens and Gurdon, 1983). Later work described a comparison of the sequences surrounding the mRNA 3' end positions of 5 genes, 2 of which produced mRNAs with 3' ends at the normal site in oocyte injection experiments and 3 of which did not (Conway and Wickens, 1985). The genes examined were the SV40 late transcription unit, the bovine growth hormone gene, the thymidine kinase gene and human α and β globin genes. The SV40 late transcription unit and the β globin gene produced mRNAs with correct 3' termini. It was noticed that these 2 genes contained a 5 base pair sequence complementary to the AATAAA sequence near the mRNA 3' end position. It was suggested that this was the reason why some of the genes produced mRNAs with correct termini and the others did not. Those precursor mRNAs with the complementary sequence might form a stem and loop structure with the site of cleavage presented in the loop and the AAUAAA and its complementary sequence forming the stem. Only if the site of cleavage was presented in this way would cleavage and polyadenylation occur. The Xenopus β globin gene has also been used in oocyte injection experiments. This gene produces transcripts in the oocyte that have a polyadenylated 3' end in the correct position (Mason et al, 1985). This gene also contains a 5 base pair sequence complementary to the AATAAA sequence near the mRNA 3' end position (Patient et al, 1983). The mouse β globin gene, at the 3' end of pAW103, also contains a sequence complementary to the AATAAA hexamer, in the region of the mRNA 3' end position (see figure 37) and as has been shown this gene also produces transcripts cleaved and polyadenylated at

the correct site. In the case of pAV103 however the complement to the AAUAAA sequence is possibly too far downstream to form the secondary structure described.

The results presented in this section demonstrated that the Xenopus oocyte was able to recognise the sequences around the mouse globin mRNA 3' end position and to produce a polyadenylated 3' end at this position on pAV103 transcripts. The basis for an assay system to investigate the sequences responsible for 3' end formation of a polyadenylated mRNA, in this case mouse globin mRNA, was established. In the next section attempts to utilise this assay system are described.

3.6 3' PROCESSING OF MOUSE GLOBIN AND XENOPUS HEAT SHOCK PRECURSOR mRNAs IN THE OOCYTE NUCLEUS

The results presented in the previous chapter demonstrated that mechanisms exist in the Xenopus oocyte nucleus that are able to generate the 3' end of mouse globin mRNA. The oocyte was therefore a potential experimental system for investigating the sequences controlling globin mRNA 3' end formation. The results described in section 3. 5 were obtained using the technique of injecting DNA into the oocyte nucleus and analysing 3' end formation of mRNAs made in the oocyte. A second experimental technique to study the same mechanism was available. This technique was based on making artificial precursor mRNAs *in vitro*. These artificial precursor mRNAs are made to resemble genuine precursor mRNAs in that they contain extra downstream sequence at their 3' end. These precursors can be injected into the oocyte nucleus and their 3' processing examined. The effect of sequence changes on the efficiency of 3' processing can then be studied. This technique has been used sucessfully to investigate 3' end formation of histone mRNAs. It has been shown that the chicken H2B histone gene (Krieg and Melton, 1984), the sea urchin H2A histone gene (Birchmier et al, 1984) and the Xenopus H4 and H1 histone genes (Georgiev et al, 1984 ; earlier chapters of this thesis) can all be used to make precursor mRNAs that are 3' cleaved after injection into the oocyte nucleus. It was considered that the approach of using artificial precursor

mRNAs had a number of advantages over DNA injection experiments for a study of mouse globin mRNA 3' end formation. Some of these advantages were as follows :

1. The result of sequence changes made directly in a precursor mRNA are more straightforward to interpret than the results of changes in the DNA sequence of a gene used for injection. For example changing the sequence of a gene for injection may have effects at the transcriptional level as well as effects on processing.

2. By using a labelled precursor mRNA 3' cleavage can be examined directly by examining changes in size of the precursor mRNA using acrylamide gel electrophoresis and autoradiography.

3. Using the precursor mRNA injection method the duration of experiments could be reduced. In a precursor mRNA injection experiment processing could be detected in 2 - 3 hours whereas in a DNA injection experiment the injected oocytes had to be incubated for 15 - 20 hours before sufficient RNA accumulated to analyse.

After using the DNA injection technique to establish that oocytes could 3' process globin mRNAs a further study to investigate the sequences necessary for processing was attempted. This further study was based on the injection of artificial globin precursor mRNAs.

A further advantage of the precursor mRNA injection method was that the fates of labelled precursor mRNAs could be followed directly without the use of labelled probes.As a consequence of this it was possible to investigate the 3' processing of Xenopus

polyadenylated mRNA using this system. This study could not have been attempted by DNA injection, with a Xenopus gene expressed in the oocyte. The reason for this was that mRNAs made in the oocyte, from the injected gene, had to be analysed indirectly using radioactive probes, these probes detected the endogenous oocyte transcripts as well as transcripts from the injected gene. It was preferable to study 3' processing of a mRNA in a homologous system, for this reason the Xenopus heat shock gene hsp70A was used to make precursor mRNA. The heat shock precursor mRNA was tested for 3' processing in the oocyte nucleus. The hsp 70A gene is constitutively expressed in the oocyte (Bienz and Gurdon, 1982) and it was expected that the oocyte nucleus would contain comparatively large amounts of the factor(s) needed to process this transcript.

b. Results

Details of the SP6 sub - clones of the mouse globin and heat shock genes are shown in figures 38, 39 and 40. Two constructs were made using the mouse β globin gene. Each construct contained a fragment spanning the mRNA 3' end position of the globin gene, cloned downstream of the SP6 promoter. pSP64.MG1 contained a 392 Sau 3A fragment comprised of 246 nt of sequence upstream of the mRNA 3' end position and 146 nt of sequence downstream from this position. When linearised with Eco RI and transcribed using SP6 polymerase this sub - clone generated a 444 nt long precursor mRNA. The extra sequence comprised of SP6 polylinker at both ends

of the globin sequence. The size of HG1 precursor mRNA cleaved at the mRNA 3' end position would be 280 nt.

pSP64.HG2 contained a 1307 nt Hind III / Xba I fragment from the globin gene. This comprised 707 nt of sequence from upstream of the mRNA 3' end position and 600 nt of sequence from downstream of this position. After linearisation using Eco RI and transcription using SP6 polymerase this sub - clone generated a precursor mRNA 1337 nt long. The size of HG2 precursor cleaved at the mRNA 3' end position was 714 nt.

pSP65.HS1 contained a 1160 Sst I / Pst I fragment from the hsp 70A gene. This fragment contained 600 nt of sequence upstream of the mRNA 3' end position and 560 nt of sequence downstream of this position. On linearisation with Hind III and transcription with SP6 polymerase this sub - clone generated a 1169 nt precursor mRNA. The size of HS1 precursor mRNA cleaved at the mRNA 3' end position would be 620 nt.

In each case described above the size of the 3' processed RNA is given as the size of the precursor mRNA after cleavage at the mRNA 3' end position. If 3' cleavage and polyadenylation occured the sizes of RNA detected would be 100 - 200 nt longer that the sizes of the 3' cleaved precursors, depending on the length of the poly - A tail added.

Each SP6 sub - clone was linearised using the appropriate restriction enzyme and transcribed in the presence of a ^{32}P rGTP and a cap analogue, this generated a full length, capped and labelled precursor mRNA. Each sub - clone also produced smaller transcripts assumed to be caused by premature transcription

termination by the SP6 polymerase. No attempt was made to separate the smaller transcripts from the full - length precursor mRNA. The precursor mRNAs were tested for 3' processing in the oocyte nucleus. In each case a parallel injection experiment was performed using the H4 precursor mRNA (described in previous sections). Each RNA was injected into the nucleus of about 20 oocytes. 10 oocytes were homogenised immediately after injection and 10 oocytes were incubated for 3 - 5 hours before homogenisation. Total RNA was made from both time point samples and precursor mRNAs were analysed for size changes using acrylamide gel electrophoresis. Each track of the electrophoresis gels contained RNA from an equal number of injected oocytes so that the tracks were comparable. After electrophoresis the positions of the precursor mRNAs and their derivatives were detected by autoradiography. The results of these experiments are shown in figure 41.

Figure 41.a Shows the results of the H4 and MG2 precursor mRNA injection experiments. In these experiments 3 time points : 0, 3 and 18 hours after injection were used. The H4 precursor mRNA was 762 nt long on injection and over the course of the experiment was converted to the 446 nt 3' cleaved form. The MG2 sub - clone generated 3 precursor mRNAs on SP6 transcription. Only the full - length precursor mRNA and the longest of the prematurely terminated RNAs contained extensions past the globin mRNA 3' end position and so only these two RNAs could have been substrates for a genuine RNA 3' processing activity. The position of 3' cleaved MG2 precursors at 714 nt is indicated. No band was

seen to appear at this position. No discrete bands approximately 100 - 200 nt longer than 714 nt, caused by 3' cleavage followed by polyadenylation, appeared either. The intensity of all 3 MG2 precursor mRNAs relative to each other also remained unaltered. These results suggested that MG2 precursor mRNAs were not 3' cleaved and polyadenylated after injection into the oocyte nucleus.

Figure 41.b Shows the result of H4 and MG1 precursor mRNA injections. Again the H4 precursor mRNA was 3' cleaved and reduced in size from 762 nt to 446 nt. The MG1 sub - clone produced 2 transcription products, one full length 444nt precursor mRNA and a shorter 220 nt RNA. Only the 444 nt precursor mRNA could have been a substrate for a 3' processing activity acting at the globin mRNA 3' end position. After injection into oocytes both MG1 RNAs were degraded to a certain extent. The shorter 220 nt RNA appeared to be slightly more unstable than the full length 444 nt RNA. The position of MG1 transcripts cleaved at the globin mRNA 3' end position, to a size of 280 nt, is shown. Over the course of the experiment no RNA of this size was formed. Furthermore no discrete RNA bands caused by 3' cleavage at the globin mRNA 3' end position followed by the addition of a 100 - 200 nt poly - A tail were produced. This strongly suggested that the MG1 transcripts were not substrates for 3' cleavage and polyadenylation after injection into the oocyte nucleus.

Figure 41.c Shows the result of H4 and HS1 precursor mRNA injections. The H4 precursor mRNA was 3' cleaved in the oocytes

to generate the 446 nt RNA. In this particular experiment a small amount of 3' cleaved H4 RNA was present in the first time point sample. This time point sample was taken after all the oocytes had been injected. By this time the first oocytes to be injected had been incubated for about 15 minutes. During this time, in this particular batch of oocytes, a small amount of H4 3' cleavage occured. Transcription of pSP65.HS1 produced 2 precursor mRNAs both of which had extensions downstream of the heat shock mRNA 3' end position. Both of these heat shock precursor mRNAs could have been substrates for a mRNA 3' processing activity. The position of 3' cleaved HS1 precursors at 620 nt is indicated. No RNA band appeared at this position over the course of the experiment. No other discrete RNA bands, 100 - 200 nt longer than 620 nt, indicative of 3' cleavage followed by polyadenylation, were produced, this strongly suggested that HS1 precursor mRNAs were not substrates for 3' cleavage and polyadenylation after microinjection into the oocyte nucleus.

In all these experiments the assumption was made that if a precursor mRNA underwent 3' processing a new discrete RNA band would be detected on electrophoresis. This would be the case if 3' processing consisted only of 3' cleavage without a subsequent polyadenylation reaction, the experiments using the histone precursor mRNAs clearly demonstrate this. If 3' cleavage was followed by a polyadenylation reaction in which all the RNA molecules gained A tails of similar length then again a new discrete RNA band would have been formed, in this case the tightness of the band would depend on the variation in the

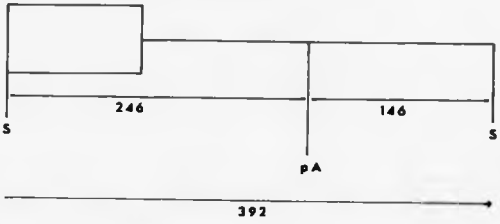
lengths of the A tails. If however 3' cleavage was followed by a polyadenylation reaction which added A tails varying very widely in length then the precursor mRNA would be converted to a form which would run as a broad smear on gel electrophoresis. This type of polyadenylation reaction was not expected and on inspection of the autoradiographs shown in figure 41 no RNA smears present only at the second time point were detected. To be certain that 3' cleavage followed by addition of heterologous length poly - A tails did not occur further experiments were carried out. In these experiments, based on S1 nuclease analysis, RNA samples from the 2 time points were hybridised to an unlabelled DNA probe homologous to the precursor mRNA. The hybrids were treated with S1 nuclease and the RNA fragments protected by the probe were analysed by acrylamide gel electrophoresis. The probe was choosen to overlap the mRNA 3' end position in the precursor mRNA. RNA that was not cleaved at this position was protected for the full length of the probe. RNA that was cleaved at the mRNA 3' end position was only protected from the 5' end of the probe to the site of 3' cleavage. 3' cleaved RNAs therefore gave shorter protected fragments after S1 nuclease treatment and gel electrophoresis. Any RNA molecule cleaved at the mRNA 3' end position, polyadenylated or not, would give the same size protected fragment because the A tail was not complementary to the probe. This technique was therefore a means of detecting 3' cleaved precursor mRNAs even if they had subsequently been subject to a polyadenylation activity adding varying length poly - A tails. The technique was tested using H4

precursor and H4 3' cleaved RNAs. The RNA samples used in these test assays were total RNAs from microinjected oocytes containing either only labelled H4 precursor mRNA or labelled 3' cleaved H4 RNA. These RNAs are shown in figure 42. Amounts of RNA from equal numbers of injected oocytes were then used in a test assay. The unlabelled DNA probe was a 463 nt Ava I fragment prepared from the pSP64.X1H4 sub - clone. This fragment extended from a Ava I site 153 nt upstream of the mRNA 3' end position to an Ava I site in the SP6 polylinker downstream of the H4 insert (figure 43). The probe was hybridised to the RNA samples at 3 different temperatures. After S1 nuclease treatment of the hybrids the protected RNA fragments were analysed by gel electrophoresis (figure 44). There was only a small amount of variation between the 3 temperatures. In the sample in which the full - length precursor mRNA was predominant a strong band 463 nt in length was present. This band was formed by protection of the RNA by the full length of the DNA probe. In the samples where 3' cleaved H4 RNA was predominant a strong band of 153 nt was present. This band was caused by protection of the RNA from the Ava I site at the 5' end of the probe to the site of cleavage at the mRNA 3' end position. The same technique was then used on MG1 and MG2 RNAs from microinjected oocytes. In this case the probe used was the 392 Sau 3A fragment spanning the globin mRNA 3' end position. The probe was prepared from the pSP64.MG1 sub - clone (figure 38). The DNA probe was then hybridised to RNA from an equal number of oocytes previously injected with either MG1 or MG2 precursor mRNAs. Samples from oocytes immediately after injection and from

oocytes 3 hours after injection were tested (figure 45). In all 4 samples the predominant band was 392 nt in length, caused by protection of the precursor mRNA by the full length of the DNA probe. Precursor mRNAs that had been cleaved at the globin mRNA 3' end position, whether polyadenylated or not, would have been protected by the probe up to the site of cleavage, this would have resulted in a 246 nt protected fragment. No band of 246 nt was detected in either the MG1 or MG2 second time point RNA samples. This result confirmed that neither MG1 or MG2 precursor mRNAs underwent 3' cleavage after injection into the oocyte nucleus. Both MG1 and MG2 samples contained shorter protected bands (labelled S on figure 45). These bands arose by protection of prematurely terminated transcripts made during the SP6 transcription. The size of the bands is a measure of the distance between the 5' end of the probe and the site of premature transcription termination, this allowed the termination site to be accurately determined (see appendix). These short RNAs possibly appear as broad bands because this premature transcription termination is not precise. The short RNAs appear to decrease in size over the course of the experiment. The site of premature transcription termination is just downstream of a potential stem / loop structure in the precursor mRNA. It may be that the observed decrease in size was due to a 3' exonuclease activity that removed some of the 3' end of the RNA but was blocked by a stem / loop structure forming in the precursor mRNA.

Fig. 38

A. 392 bp S fragment from pAW103



B. Cloned in pSP64

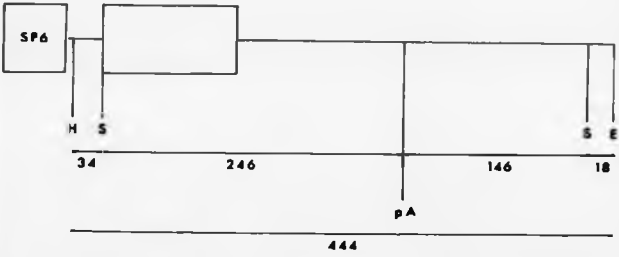


Figure 38. The mouse globin DNA fragment cloned in pSP64.MG1

Figure 38.A The 392 Sau 3A fragment spanning the mouse β globin mRNA 3' end position (pA). This fragment contained 246 nt of sequence from upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of this position.

Figure 38.B The 392 nt Sau 3A fragment cloned into the Bam HI site of pSP64. When linearised with Eco RI and transcribed with SP6 polymerase this sub - clone generated a 444 nt full length precursor mRNA. The precursor mRNA comprised of the globin sequence plus small amounts of SP6 polylinker sequence.

SP6 = SP6 promoter.

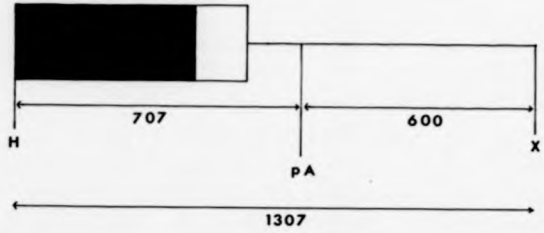
Key to restriction enzyme abbreviations :

H = Hind III S = Sau 3A E = Eco RI

All sizes marked are in nucleotides.

Fig.39

A. 1307 bp H/X fragment from pAW103



B. Cloned in pSP64

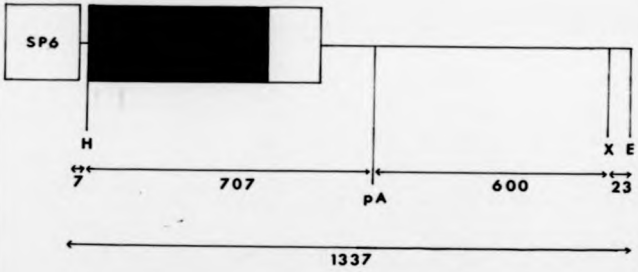


Figure 39. The globin DNA fragment cloned in pSP64.MG2

Figure 39.A. The 1307 nt Hind III / Xba I fragment from pAW103 spanning the globin mRNA 3' end position (pA).

Figure 39.B. The Hind III / Xba I globin fragment cloned in the Hind III and Xba I sites of pSP64.

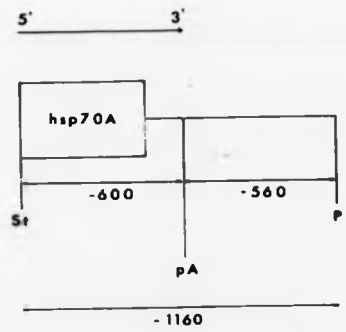
The blacked out area of the globin gene represents an intron sequence.
SP6 = SP6 promoter.

Key to abbreviations of restriction enzyme sites :

H = Hind III X = Xba I E = Eco RI

Fig.40

A. The 3' end of the Xenopus hsp70A gene



B. Cloned in pSP65

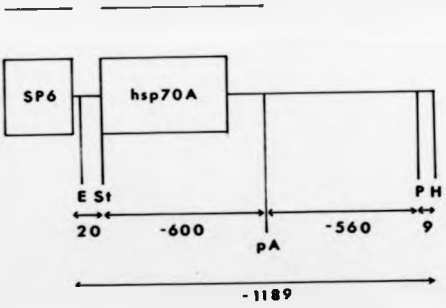


Figure 40. The hsp70A DNA fragment cloned in pSP65 HS1

Figure 40.A. The Sst I / Pst I restriction enzyme fragment spanning the mRNA 3' end position (pA) of the Xenopus hsp70A gene.

Figure 40.B. The 1160 nt Sst I / Pst I hsp70A fragment cloned in the Sst I and Pst I restriction sites of pSP65, this made the sub - clone pSP65.HS1.

Key to abbreviations of restriction enzyme sites :

St = Sst I P = Pst I E = Eco RI H = Hind III

All sizes shown are in nucleotides.

Fig. 41

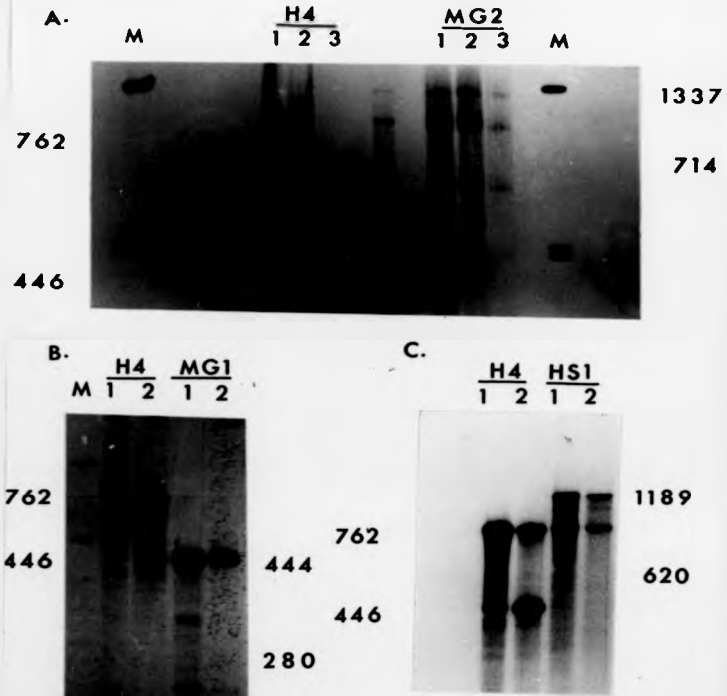

A.
M
H4
1 2 3
MG2
1 2 3
M
1337
762
714
446
B.
M
H4
1 2
MG1
1 2
762
446
444
280
220
C.
H4
1 2
HS1
1 2
762
446
1189
620

Figure 41. Microinjection of MG1, MG2 and HS1 precursor mRNAs into the oocyte nucleus

Radioactively labelled H4, MG1, MG2 and HS1 precursor mRNAs were injected into the oocyte nucleus. RNA samples were prepared from oocytes immediately after injection (1) and 3 hours after injection (2). In some experiments a further RNA sample was prepared from injected oocytes approximately 18 hours after injection (3). The RNA samples were analysed by acrylamide gel electrophoresis.

Figure 41.A. Injection of H4 and MG2 precursor mRNAs into the oocyte nucleus.

M = pBR322 Hinf I size marker, 762 nt = Size of H4 precursor mRNA, 446 nt = Size of 3' cleaved H4 RNA, 1337 nt = Size of full length MG2 precursor mRNA, 714 nt = Size of 3' cleaved MG2 RNA.

Figure 41.B. Injection of H4 and MG1 precursor mRNAs into the oocytes nucleus.

M = pBR322 Hinf I size marker, 762 nt = Size of H4 precursor mRNA, 446 nt = Size of 3' cleaved H4 RNA, 444 nt = Size of full length MG1 precursor mRNA, 280 nt = size of 3' cleaved MG1 RNA, 220 nt = Size of prematurely terminated MG1 transcript.

Figure 41.C. Injection of H4 and HS1 precursor mRNAs into the oocyte nucleus.

762 nt = Size of H4 precursor mRNA, 446 nt = Size of 3' cleaved H4 RNA, 1189 nt = Size of full length HS1 precursor mRNA, 620 nt = Size of 3' cleaved HS1 RNA.

Fig. 42

Figure 42. H4 RNA samples used for optimisation of S1 assay conditions

RNA samples from injected oocytes containing either only radioactively labelled H4 precursor mRNA or only radioactively labelled 3' cleaved H4 RNA were used to optimise the conditions for an S1 nuclease assay. This S1 nuclease assay used an unlabelled DNA probe to map the 3' ends of radioactively labelled H4 precursor mRNAs (See figure 44).

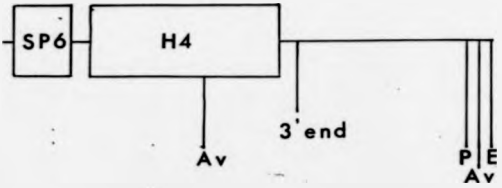
1 = RNA sample from injected oocytes containing only labelled H4 precursor mRNA.

2 = RNA sample from injected oocytes containing only labelled 3' cleaved H4 RNA.

762 nt = Size of H4 precursor mRNA.

446 nt = Size of 3' cleaved H4 RNA.

Fig. 43



453 nt Ava 1 probe

153 nt from Ava 1 site to mRNA 3' end position

Figure 43. The DNA probe used for S1 mapping the 3' ends of labelled pSP64.X1H4 transcripts

pSP64.X1H4 DNA was digested with Ava I and the 453 nt Ava I fragment spanning the H4 mRNA 3' end position was isolated. This fragment was then used as an unlabelled probe in S1 analysis of labelled pSP64.X1H4 transcripts. For transcription pSP64.X1H4 was linearised using Eco RI.

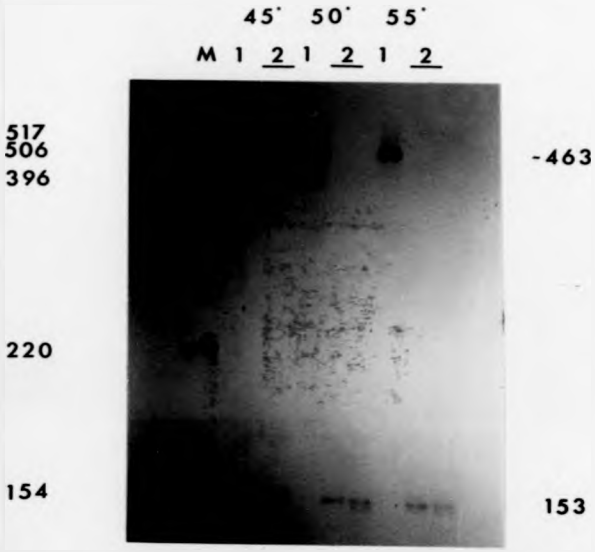
Key to abbreviations of restriction enzyme sites :

Av = Ava I P = Pst I E = Eco RI

3' end = Histone H4 mRNA 3' end position.

SP6 = SP6 promoter

Fig. 44


45' 50' 55'
M 1 2 1 2 1 2
517
506
396
220
154
-463
153

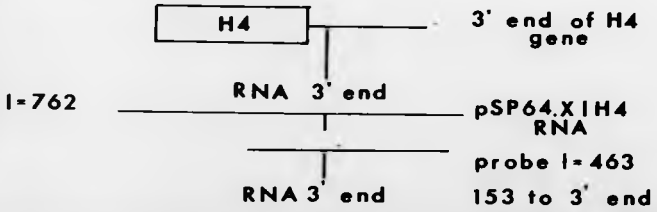

H4
3' end of H4 gene
RNA 3' end
1=762
pSP64.X I H4 RNA
probe 1=463
RNA 3' end
153 to 3' end

Figure 44. Optimisation of S1 assay conditions for the use of an unlabelled DNA probe to map the 3' ends of radioactively labelled H4 RNAs

Samples from injected oocytes containing either radioactively labelled H4 precursor mRNA (1) or radioactively labelled 3' cleaved H4 RNA (2) were used to optimise the conditions for an S1 nuclease assay using an unlabelled DNA probe to map the 3' ends of H4 transcripts. The probe used was a 463 nt Ava I fragment prepared from pSP64.X1H4. The probe contained 153 nt of sequence upstream of the H4 mRNA 3' end position and 310 nt of sequence downstream of this position. This probe was hybridised to the radioactive RNA samples at 3 different temperatures, after S1 nuclease treatment the fragments of the radioactive RNAs protected by the probe were analysed by acrylamide gel electrophoresis.

M = pBR322 Hinf I size marker.

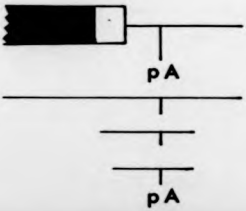
1 = Analysis of RNA samples from injected oocytes containing labelled H4 precursor mRNA.

2 = Analysis of RNA samples from injected oocytes containing labelled 3' cleaved H4 RNA.

463 nt = Size of uncleaved H4 precursor mRNA protected for the full length of the probe.

153 nt = Size of H4 RNA 3' cleaved at the histone mRNA 3' end position and protected by the probe up to this position.

Fig. 45



3' end of globin gene

MG2 1337

MG1 444

probe 392

246 to pA

Figure 45. S1 nuclease analysis on RNAs from oocytes injected with radioactive MG1 and MG2 precursor mRNAs, using an unlabelled DNA probe.

Radioactively labelled MG1 and MG2 precursor mRNAs were injected into the nucleus of Xenopus oocytes. RNA samples were prepared from oocytes immediately after injection (1) and from oocytes 3 hours after injection (2). These RNA samples were subject to an S1 nuclease analysis using an unlabelled DNA probe. The probe was a 392 nt Sau 3A fragment that spanned the globin mRNA 3' end position present in MG1 and MG2. The DNA probe contained 246 nt of sequence from upstream of the globin mRNA 3' end position and 146 nt of sequence from downstream of this position.

M = pAT153 hinf I marker.

MG1 1 and MG1 2 = S1 analysis on RNAs from oocytes injected with MG1 precursor mRNA.

MG2 1 and MG2 2 = S1 analysis on RNAs from oocytes injected with MG2 precursor mRNA.

392 nt = Protection of MG1 and MG2 Precursor mRNAs by the full length of the DNA probe.

246 nt = Expected position of RNA fragment protected by the probe from 3' cleaved MG1 and MG2 RNAs.

S = Protection of prematurely terminated MG1 and MG2 transcripts by the DNA probe.

c. Discussion

The results presented in this chapter have demonstrated that 2 mouse globin precursor mRNAs and a Xenopus hsp 70A precursor mRNA do not undergo 3' processing, to any detectable level, after injection into the oocyte nucleus. The assays used to detect 3' processing were sufficiently sensitive to detect 3' cleavage of a histone H4 precursor mRNA, therefore if 3' processing of globin or heat shock precursor mRNAs did occur it was very inefficient compared to histone 3' processing. No detectable 3' processing of globin or heat shock precursor mRNAs was detected despite the fact that the oocyte possesses mechanisms that can form the 3' ends of these transcripts in certain circumstances. This ability was demonstrated, in the case of the globin mRNA 3' end, by the experiments described in section 3. 5 in which pAW103 DNA was injected into the oocyte nucleus. The oocyte must also possess a mechanism to generate the 3' ends of its endogenous heat shock mRNAs. Possible explanations for the anomaly between DNA and RNA injection experiments for the globin mRNA 3' end and for the lack of processing of the heat shock precursor mRNA are of 2 types. Explanations may be specific and seek to explain specifically why the 3 precursor RNAs used in these experiments were not processed or general and seek to explain why injection of a general SP6 precursor mRNA does not lead to its 3' processing.

All the precursor mRNAs used in these experiments were capped as they were made. Based on the results from the histone precursor mRNA injection experiments a capped precursor mRNA would be expected to be 3' processed as long as all the sequences

necessary for processing were present. The most obvious specific explanation for the lack of 3' processing is that MG1, MG2 and HS1 precursor mRNAs lacked sequences necessary for processing. From previous work it is clear that sequences upstream of the AAUAAA hexamer are not required for mRNA 3' end formation, this was recently demonstrated in experiments in which the Xenopus β globin gene was injected into the oocyte nucleus. In these experiments it was shown that sequences to within 9 nt upstream of the AAUAAA hexamer could be deleted from the mRNA 3' untranslated region without affecting mRNA 3' end formation (Mason et al, 1986). Sequences downstream of the mRNA 3' end position have however been implicated in mRNA 3' end formation. These downstream sequences are discussed in detail in the thesis introduction. The downstream sequences implicated in mRNA 3' end formation are relatively short distances downstream from the mRNA 3' end position. In none of the experiments to date have sequences more that about 80 nt downstream of the mRNA 3' end position been implicated in mRNA 3' end formation. MG1, MG2 and HS1 precursor mRNAs contained 146, 600 and 560 nt of sequence past the mRNA 3' end position respectively. All of the precursor mRNAs should therefore have contained the sequences necessary for 3' end formation. For the mouse globin gene, in erythroleukemia cells, it has been shown that transcription termination occurs in a region begining about 700 nt downstream from the mRNA 3' end position (Citron et al, 1984). This region was not present in the MG1 and MG2 precursor mRNAs. It is possible that a sequence in this region is necessary for 3' processing, perhaps by forming

a particular secondary structure in the precursor mRNA. Related to this point it has been shown that transcription of the SV40 late transcription unit continues in the downstream direction past the mRNA 3' end position (Ford and Hsu, 1978). It has also been shown that a 220 nt DNA fragment spanning the SV40 late mRNA 3' end position contains sufficient sequences to direct mRNA 3' end formation in oocyte microinjection experiments. This 220 nt fragment contained only 79 nt of sequence past the mRNA 3' end position and presumably did not include the region where the majority of transcription termination occured in the wild type gene (Conway and Wickens, 1985). In this case 3' end formation in the oocyte did not require a specific sequence associated with a transcription termination region. The same is very likely to be the case for the MG1 and MG2 precursor mRNAs.

An alternative specific explanation is that MG1, MG2 and HS1 RNAs contained sequences which were inhibitory to mRNA 3' end formation. None of these precursor mRNAs contained extra sequences from the relevant genes which could not have been present in genuine precursor mRNAs. However all 3 precursors contained sequences from the SP6 polylinker at both the immediate 5' and 3' ends. The amount of extra sequence between the precursor mRNA cap and the specific gene sequences ranged from 7 nt in MG2 to 34 nt in MG1. The amount of extra polylinker sequence directly at the 3' end of the precursor mRNAs ranged from 9 nt in HS1 to 23 nt in MG2. The extra 5' sequences would be very unlikely to have any affect on mRNA 3' end formation. The extra 3' sequences would be more likely to be inhibitory

especially if the mechanism of 3' processing involved a processive exonuclease activity. *In vitro* 3' processing involves an endonucleolytic cleavage reaction for adenovirus and SV40 mRNAs (Moore and Sharp, 1985 ; Zarkower et al, 1986). In addition extra polylinker sequence at the 3' end of H4 precursor mRNAs does not inhibit 3' cleavage in oocyte injection experiments. It was concluded that neither the absence of specific sequences or an inhibitory affect by SP6 polylinker sequences were adequate explanations for the observed lack of processing of MG1, MG2 and HS1 precursor mRNAs.

It is more likely that a general explanation relating to the experimental procedure of preursor mRNA injection is the correct one . To my knowledge no experiments have been reported in which precursor mRNAs to polyadenylated mRNAs have been shown to undergo efficient 3' processing after injection into the oocyte nucleus. It would be suprising, given the sucess of histone precursor mRNA injection experiments, if the same technique had not been attempted for polyadenylated mRNAs. The only mention in the literature of the precursor mRNA injection method being used to investigate 3' processing of a polyadenylated mRNA was experiments in which precursors to the human ß globin gene were injected into oocytes (Green et al,1983). It was claimed that accurate but inefficient 3' cleavage at the mRNA 3' end position of these precursors did occur after they were injected into the oocyte nucleus. It was possible that in my experiments very inefficient 3' cleavage occured but this was not detected.

One such general explanation for the inefficient 3' processing of precursors polyadenylated mRNAs is that although the precursors were injected into the oocyte nucleus they were transported out of the nucleus before processing could occur. This was not the case for the H4 precursor mRNA and there is no reason to believe that this precursor mRNA would be transported out of the nucleus at a different rate to the MG1, MG2 and HS1 precursor mRNAs.

A second general explanation could be that 3' processing of polyadenylated mRNAs requires a specific structure which forms as the precursor mRNA is transcribed. The artificial precursor mRNAs injected into the oocytes lack this structure and therefore do not undergo 3' processing. This sort of idea was first proposed to explain the lack of processing of artificial precursor mRNAs added to a HeLa whole cell *in vitro* processing system (Moore and Sharp, 1984). It has however since been shown that exogenously synthesised precursor mRNAs can be 3' processed when added to improved *in vitro* processing systems (Moore and Sharp, 1985). This explanation might be correct for the oocyte system but there is no evidence in its favour.

A third general explanation is the one that I consider to be the most likely. This is related to the fact that in the precursor mRNA injection experiments the oocyte is suddenly presented with a relatively large amount of precursor mRNA. If 3' processing to a polyadenylated mRNA 3' end requires more than 1 factor to bind to the precursor mRNA, injecting a large amount of precursor mRNA may saturate the system. In this case few of the

precursor mRNA molecules would bind sufficient factors to undergo 3' processing. The factors involved could be low abundance snRNPs. This explanation could also explain the observed difference between the globin DNA and RNA injection experiments. In the DNA injection experiments globin precursor mRNAs were presented to the oocyte initially at low levels as the injected DNA was transcribed, for this reason the oocytes processing system was not saturated and transcripts 3' processed at the globin mRNA 3' end position were produced. Under similar experimental conditions injected H4 precursor mRNAs were 3' cleaved, this might have been because only 1 factor was necessary for histone mRNA 3' end formation or because the processing factors required were more abundant.

OVERALL COMMENTS

The work presented in the first section of the thesis set out to investigate whether changes in histone mRNA 3' cleavage could account for changes in the rate of production of histone proteins during early Xenopus development. During oogenesis histone protein is produced at the low constant rate of 40 pg / hour, when the oocyte matures into an egg the rate increases to 2.5 ng / hour and in the blastula embryo the rate is about 7 ng / hour (Adamson and Woodland, 1974). The increase in production of histone protein, after oocyte maturation, occurs in the absence of the production of new histone mRNA which does not begin until the mid - blastula transition (Newport and Kirschner, 1982b). The population of histone transcripts accumulated in the oocyte support the increase in histone protein production in the early embryo. One way in which this could happen would be for some change to the existing mRNAs to occur to make them be translated more efficiently. A second explanation could be that there is an increase in the amount of histone mRNA available for translation in the egg and the embryo over the oocyte. The increase in the amount of translatable histone mRNA could be achieved by the accumulation, in the oocyte, of a stockpile of histone precursor mRNAs that are unprocessed at the 3' end. These unprocessed precursors would be 3' cleaved and translated in the egg and early embryo. This hypothesis was investigated in 2 ways : firstly artificial histone precursor

mRNAs were microinjected into oocytes, eggs and embryos to determine whether all these developmental stages possessed mechanisms to 3' cleave histone precursor mRNA, secondly Northern analysis, using a histone probe, was performed on RNAs from oocytes and eggs to determine whether histone precursor mRNAs, longer than mature histone transcripts, did exist in these developmental stages.

The results from the histone precursor mRNA injections demonstrated that matured oocytes, eggs and embryos all possessed mechanisms to 3' cleave histone precursor mRNAs. The 3' cleavage mechanism was present in these stages despite the fact that it would not be required to 3' cleave newly transcribed histone precursor mRNA. The histone mRNA 3' cleavage mechanism detected in these stages could be used to process histone mRNA precursors stockpiled during oogenesis. However the Northern analysis, using a histone probe, failed to detect any significant level of histone transcripts, longer than mature histone mRNAs, in either oocytes or eggs.

As a result of these experiments it was concluded that although 3' cleavage of stored histone precursors could occur in the egg and the embryo it probably is not an important mechanism in the increase in histone protein synthesis. It is probable that changes to histone mRNAs, such as modifications to the 5' cap structure or changes in adenylation status, lead to an increase in translational efficiency. The latter might be of prime importance because it has been shown that when the oocyte matures into an egg histone transcripts become de - adenylated (

Ballantine and Woodland, 1985). It is difficult to speculate as to how de - adenylation of histone mRNAs causes an increase in their translation.

Arising from the experiments on histone mRNA 3' cleavage during development a polyadenylation activity was discovered. The investigation of this activity provided the majority of the work in the first section of the thesis. The activity was present in matured oocytes, eggs and early embryos but was absent from stage 6 oocytes. The activity added an A tail, approximately 60 - 70 A residues, long to Xenopus H4, H1 and heat shock precursor mRNAs. RNAs made from the mouse β globin gene were not substrates for the activity. The possibility that the activity was controlled by the AAUAAA hexamer was considered. The H1 precursor mRNA contained a copy of the AAUAAA hexamer close to its 3' end. When the 3' end of the H4 fragment, that formed the 3' end of the H4 precursor mRNA, was sequenced, 2 sequences with 5 out of 6 nucleotides identical to the AAUAAA hexamer were identified. One of these sequences might have been responsible for controlling polyadenylation of the H4 precursor mRNA. One argument against the idea that the polyadenylation activity was controlled by the AAUAAA hexamer, or a similar sequence, was that all 3 transcripts from the heat shock SP6 sub - clone were polyadenylated after microinjection into matured oocytes. These heat shock transcripts included the full length precursor mRNA and 2 prematurely terminated RNAs. It was considered unlikely that all 3 of the heat shock RNAs would have had an AAUAAA hexamer, or similar sequence, sufficiently near the 3' end to direct the

polyadenylation activity. The sequence of the downstream region from the Xenopus hsp70A gene was not available. One obvious further step would be to sequence this region, identify the sites of the 3' ends of the prematurely terminated hsp70A precursor mRNAs and examine the sequences upstream of these sites for copies of the AAUAAA hexamer. Experiments to investigate the importance of the AAUAAA hexamer in polyadenylation of the H4 and H1 precursor mRNAs could also be performed. The AAUAAA hexamer in the H1 precursor mRNA, and the similar sequences in the H4 precursor mRNA, could be removed by deletion. The deleted precursor mRNAs could then be tested for polyadenylation in microinjected matured oocytes, eggs or embryos. A further possibility was that a sequence unlike AAUAAA was present in the precursor mRNAs and that this sequence controlled the polyadenylation activity. Deletion experiments to progressively remove sequences from the 3' ends of the precursor mRNAs could be performed to investigate this possibility. A third possibility was that the polyadenylation activity was a general effect and that the globin precursor mRNAs contained a sequence that was inhibitory to this effect. Deletions to progressively remove sequences from the 3' end of the mouse globin precursor mRNAs and the testing of the deleted RNAs for polyadenylation could be performed to test this possibility.

The extra experiments discussed above would provide information as to the mechanism of the polyadenylation activity but they would not provide insights into the possible significance of the activity during early Xenopus development.

The activity was discovered because it acted on histone precursor mRNAs microinjected into eggs and early embryos, however the activity is unlikely to play a physiological role in the metabolism of histone mRNA. In the stages where the activity was shown to exist the endogenous Xenopus histone mRNAs are de - adenylated rather than becoming adenylated. The polyadenylation activity is more likely to be an activity that is important in the metabolism of non - histone mRNAs. Polyadenylation changes during oocyte maturation, and after fertilisation, are known to occur (see introduction). However the data is sparse and no coherant model for the role, if any, of these changes has been postulated. The work described in this thesis has demonstrated that a very active polyadenylation activity is present in microinjected matured oocytes, eggs and embryos, a thorough study to determine changes in the polyadenylation status of known transcripts and how these changes affect the future translatability and stability of the transcripts would be necessary before the significance of the activity could be evaluated.

The work presented in the first section of the thesis was sucessful in answering the original question of whether changes in histone mRNA 3' cleavage might play a role in controlling the rate of production of histone proteins during early development. Only a small number of relatively simple experiments were necessary to investigate this point, the discovery of the polyadenylation activity and its subsequent investigation provided the bulk of the section.

The aim of the work described in the second section of the thesis was to develop an experimental system, based on oocyte microinjection, to investigate 3' end processing of a polyadenylated mRNA. The main reason for the choice of the oocyte as an experimental system was the success of this system in elucidating the mechanisms of 3' end formation of non - adenylated histone transcripts. In addition when the work was started *in vitro* systems, with the ability to form the 3' ends of polyadenylated mRNAs, were not available.

pAW103 DNA was microinjected into the oocyte nucleus to investigate 3' end formation of mouse β globin mRNA. The results demonstrated that the oocyte has the ability to form the mouse globin mRNA 3' end at the correct site and to polyadenylate the mRNA. No pAW103 transcripts aberrantly processed at the 3' end were detected. It had originally been expected that 3' processing of pAW103 transcripts might be aberrant in the oocyte, if this had been the case complementation experiments were planned in order to try to identify a factor from mouse cells that could complement the lesion in 3' processing. Because pAW103 mRNA 3' processing was accurate in injected oocytes these experiments were ruled out. It appeared that in order to exploit the system in any way deletion experiments, to identify sequences involved in 3' processing, were necessary.

When the SP6 *in vitro* transcription system became available it was decided to use this system, in conjunction with oocyte microinjection, to investigate the sequences necessary for globin

mRNA 3' processing. It was felt that injection of globin precursor mRNAs, and globin precursor mRNAs with deleted sequences, had a number of potential advantages over DNA injection (see section 3.6). However when 3 different globin precursor mRNAs were injected into the oocyte nucleus none were found to undergo 3' processing. The possible explanations for this lack of 3' processing are described in section 3.6. The explanation considered to be the most likely was that on injection of a comparatively large amount of precursor mRNA the oocyte's processing systems became saturated and few of the precursor mRNA molecules bound sufficient factors to be 3' processed. The lack of processing meant that obviously the precursor mRNA injection system could not be used to identify sequences necessary for processing. *In vitro* systems have now been developed that will 3' cleave and polyadenylate added precursor mRNAs (Moore and Sharp, 1985; Hart et al, 1985 b). These *in vitro* processing systems have already contributed important information on the mechanism of polyadenylated mRNA 3' end formation and they probably provide the best hope for exactly defining the minimal sequence requirements for mRNA 3' cleavage and for polyadenylation.

The finding that globin precursor mRNAs were not 3' processed after injection into the oocyte nucleus could still however prove to be very useful, if the proposed explanation for the lack of processing is the correct one. The injection of artificial globin precursor mRNAs could be used as the basis for a complementation assay, to identify processing factors from

mouse cells, much as had been originally intended for the pAW103 DNA injections. This system, in which the positive affects of added factors would be assayed, is preferable to systems in which the involvement of factors is infered from specific inhibitory effects.

This second section of the thesis has described work on the use of the oocyte nuclear microinjection technique to investigate polyadenylated mRNA 3' end formation, this could form the basis of a detailed investigation using a complementation assay approach.

Appendix

PREMATURE TRANSCRIPTION TERMINATION BY SP6 POLYMERASE

The SP6 *in vitro* transcription system was developed so that sequences, cloned downstream of the SP6 promotor, could be transcribed to produce defined artificial RNAs. The position of the 3' end of the artificial RNA is determined by cutting the SP6 sub - clone downstream of the inserted sequence. When both pSP64.X1H1 and pSP64.X1H4 were transcribed the only major product in each case was the full length run - off RNA. This was not the case when pSP64.MG1, pSP64.MG2 and pSP65.HS1 were transcribed, these sub - clones generated significant amounts of shorter RNAs in addition to the full length run - off RNA. It was assumed that the shorter RNAs were caused by the SP6 polymerase terminating transcription at specific sites within the cloned sequences. An attempt was made to localise one transcription termination site within the MG1 and MG2 sub - clones.

pSP64.MG1 contains a 392 nt Sau 3A fragment from the mouse globin gene. On transcription of linearised pSP64.MG1, 3 RNA products were made (figure 13). These RNAs were the full length run - off RNA, 444 nt in length, a low abundance transcript approximately 250 nt in length and a prominant shorter transcript approximately 215 nt in length. The sizes of these RNAs were obtained by comparison with denatured DNA markers. Approximately 50% of the MG1 transcripts were present in the 215

nt shorter RNA band. Based on the size of the 215 nt RNA an SP6 transcription terminator region was located in the globin sequence.

pSP64.MG2 contains a 1307 nt fragment from the mouse globin gene, this fragment includes the region of the globin gene cloned in pSP64.MG1. On transcription with SP6 polymerase MG2 generated 3 RNAs (figure 14 a, b and c). RNA a was the full length run - off RNA. b and c were premturely terminated transcripts. b was of a size consistent with being caused by transcription terminating downstream of the globin mRNA 3' end position in an unsequenced region of the gene. c was of a size consistent with being generated by transcription termination at the globin sequence responsible for producing the 215 nt RNA on transcription of MG1. This suggestion was tested and the termination region again located by the experiments presented in section 3. 6 (figure 45). In these experiments an unlabelled DNA probe was hybridised to labelled SP6 generated RNA and the hybrids treated with S1 nuclease. The probe used was the 392 nt Sau 3A fragment as cloned in the MG1 sub - clone. After hybridisation to MG1 RNA this probe protected 2 RNA bands. The full length MG1 RNA was protected for 392 nt, the shorter premmturely terminated RNA was protected from the 5' end of the probe to the premature termination site. When the same probe was used on MG2 transcription products both RNAs a and b were protected for 392 nt, RNA c was protected for a shorter length determined by the distance from the 5' end of the probe to the site of transcription termination. The length of protected

fragment from RNA c was the same as the length of protected fragment from the 215 nt MG1 RNA. This result confirmed that MG2 RNA c was generated as a result of transcription terminating at the same site as was involved in the generation of the 215 nt MG1 RNA. The location of the terminator region obtained from this data was cross - checked with the location obtained by the size comparison of the MG1 RNAs. The sequence of the globin SP6 transcription terminator located by the 2 methods is shown below :

```
5'                                                    3'
GAACAATGGTTAATTGTTCCCAAGAGAGCATCTGTCAGTTGTTGGCAAAA
--------    --------                  -----------
   Palindrome                          Termination
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(Sequence from Konkel et al, 1978)

The site of transcription termination, by the SP6 polymerase, is shown as a region, a precise site of premature transcription termination could not be determined.

2 explanations for premature transcription termination were considered :

1. transcription terminated at sequences rich in the nucleotide that was limiting in the transcription mix
2. transcription terminated after sequences in the sub - clone that resembled prokaryotic transcription terminators.

The first explanation is unlikely to be correct for the terminator sequence shown above. The limiting nucleotide in the transcription reactions was rGTP. The terminator region is not

particularly rich in G residues. Furthermore increasing the concentration of rGTP in the transcription mix did not reduce the proportion of prematurely terminated transcripts (data not shown).

The terminator region shown above does have some similarities to prokaryotic transcription terminators, these terminators often consist of a potential stem / loop structure followed by by an A/T rich region . In the sequence shown above the potential RNA stem / loop structure is indicated. The sequence following the stem / loop structure is not however particularly rich in A and T residues.

The presence of regions in the globin and heat shock sub - clones that directed premature termination of the SP6 polymerase was a considerable disadvantage in the use of the SP6 system to generate precursor mRNAs for RNA injection experiments. Attempts were made to reduce the proportion of prematurely terminated transcripts and after transcription to separate the full - length RNA from the other transcription products. Neither of these were particularly sucessful and the experiments were finally performed using a mixture of transcription products, this problem with the SP6 system had not been anticipated.

Reference list

Adamson, E.D. and Woodland, H.R. (1974). Histone synthesis in early amphibian development: histone and DNA syntheses are not co - ordinated. J. Mol. Biol. 88, 263 - 285.

Adesnik, M. and Darnell, J.E. (1972). Biogenesis and characterisation of histone messenger RNA in HeLa cells. J. Mol. Biol. 67, 397 - 406.

Amara, S.G., Evans, R.M. and Rosenfeld, M.G. (1984). Calcitonin / calcitonin gene related peptide transcription unit: Tissue - specific expression involves selective use of alternative polyadenylation sites. Mol. Cell. Biol. 4, 2151 - 2160.

Anderson, D.M., Richter, J.D., Chamberlin, M.E., Price, D.H., Britten, R.J., Smith, L.D. and Davidson, E.H. (1982). Sequence organisation of the poly (adenylic acid) RNA synthesised and accumulated in lampbrush chromosome stage Xenopus laevis oocytes. J. Mol. Biol. 155, 281 - 309

Ballantine, J.E.M. and Woodland, H.R. (1985). Polyadenylation of histone mRNA in Xenopus oocytes and embryos. FEBS Lett. 180, 224 - 228.

Bannon, G.A., Calzone, F.J., Bowen, J.K., Allis, C.D. and Gorovsky, M.A. (1983). Multiple, independently regulated, polyadenylated messages for histone H3 and H4 in Tetrahymena. N.A.R. 11, 3903 - 3917.

Belanger, A.M. and Schautz, A.W. (1975). Precocious activation of amphibian oocytes by divalent ionophore A23187. Dev. Biol. 45, 378 - 381.

Bendig, M.M. (1981). Persistence and expression of histone genes injected into Xenopus eggs in early development. Nature 292, 65 - 67.

Benoist, C., O'Hare, K., Breathnach, R. and Chambon, P. (1980). The ovalbumin gene - sequence of putative control regions. N.A.R. 8, 127 - 142.

Berk, A.J. and Sharp, P.A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease - digested hybrids. Cell 12, 721 - 732.

Berget, S.M. (1984). Are U4 small nuclear ribonucleoproteins involved in polyadenylation ? Nature 309, 179 - 182.

Berget, S.M. and Robberson, B.L. (1986). U1, U2 and U4/U6 small nuclear ribonucleoproteins are required for in vitro splicing but not polyadenylation. Cell 46, 691 - 696.

Bienz, M. (1984). Xenopus hsp 70 genes are constitutively expressed in injected oocytes. E.M.B.O. J. 3, 2477 - 2483.

Bienz, M. and Gurdon, J.B. (1982). The heat - shock response in Xenopus oocytes is controlled at the translational level. Cell 29, 811 - 819.

Birchmier, C., Grosschedl, R. and Birnstiel, M.L. (1982). Generation of authentic 3' termini of an H2A mRNA in vivo is dependent on a short inverted DNA repeat and on spacer sequences. Cell 28, 739 - 745.

Birchmier,C., Folk.,W. and Birnstiel,M.L. (1983). The terminal RNA stem - loop structure and 80 bp of spacer DNA are required for the formation of 3' termini of sea urchin H2A mRNA. Cell 35, 433 - 440.

Birchmier,C., Schumperli,D., Sconzo,G. and Birnstiel,M.L. (1984). 3' editing of mRNAs: sequence requirements and involvement of a 60 nucleotide RNA in maturation of histone mRNA precursors. Proc. Nat. Acad. Sci .USA 81, 1057 - 1061.

Birnboim,H.C. and Dolby,J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. N.A.R. 7, 1513 - 1523.

Birnstiel,M.L., Busslinger,M. and Strub,K. (1985). Transcription termination and 3' processing: the end is in site. Cell 41, 349 - 359.

Black,D.L., Chabot,B. and Steitz,J.A. (1985). U2 as well as U1 small nuclear ribonucleoproteins are involved in pre - mRNA splicing. Cell 42, 737 - 750.

Black,D.L. and Steitz,J.A. (1986). Pre - mRNA splicing in vitro requires intact U4/U6 small nuclear ribonucleoprotein. Cell 46, 697 - 704.

Bolivar,F.Z., Rodriguez,R.L., Greene,P.J., Betlach,M.C., Heynaker,H.L., Boyer,H.W., Crosa,J.H. and Falkow,S. (1977). Construction and characterization of new cloning vehicles, II. A multiple cloning system. Gene 2, 95 - 113.

Bringmann,P., Rinke,J., Appel,B., Reuter,R. and Luhrmann,R. (1983). Purification of snRNPs U1, U2, U4, U5 and U6 with 2,2,7-trimethylguanosine specific antibody and definition of their

constituent proteins reacting with anti - Sm and anti - (U1)RNP antisera. E.M.B.O. J. 2, 1129 - 1135.

Busch,H., Reddy,R., Rothblum,L. and Choi,C.Y. (1982). snRNAs, snRNPs and RNA processing. Ann. Rev. Biochem. 51, 617 - 654.

Busslinger,M., Portmann,R. and Birnstiel,M.L. (1979). A regulatory sequence near the 3' end of sea urchin histone genes. N.A.R. 6, 2997 - 3008.

Cabada,M.O., Darnbrough,C, Ford,P.J. and Turner,P.C. (1977). Differential accumulation of two size classes of poly (A) associated with messenger RNA during oogenesis in Xenopus laevis. Dev. Biol. 57, 427 - 439.

Calvet,J.P., Meyer,L.M. and Pederson,T. (1982). Small nuclear RNA U2 is base paired to heterogenous nuclear RNA. Science 217, 456 - 458.

Chabot,B., Black,D.L., LeMaster,D.M. and Steitz,J.A. (1985). The 3' splice site of pre - messenger RNA is recognised by a small nuclear ribonucleoprotein. Science 230, 1344 - 1349.

Citron,B., Falck - Pedersen,E., Salditt - Georgieff,M. and Darnell,J.E. (1984). Transcription termination occurs within a 1000 base pair region downstream from the poly (A) site of the mouse β - globin (major) gene. N.A.R. 12, 8723 - 8731.

Clarkson,S.G., Smith,H.O., Schaffner,W., Gross,K.W. and Birnstiel,M.L. (1976). Integration of eukaryotic genes for 5S RNA and histone proteins into a phage lambda receptor. N.A.R. 3, 2617 - 2632.

Colot, H.V. and Rosbash, M. (1982). Behavior of individual maternal pA+ RNAs during embryogenesis of Xenopus laevis. Dev. Biol. 94, 79 - 86.

Conway, L. and Wickens, M. (1985). A sequence downstream of AAUAAA is required for formation of simian virus 40 late mRNA 3' termini in frog oocytes. Proc. Nat. Acad. Sci. USA 82, 3949 - 3953.

Corden, J., Wasylyk, B., Buchwalder, A., Sassone - Corsi, P. Kedinger, C. and Chambon, P. (1980). Promoter sequences of eukaryotic protein coding genes. Science 209, 1406 - 1414.

Darnbrough, C. and Ford, P.J. (1979). Turnover and processing of poly (A) in full - grown oocytes and during progesterone induced oocyte maturation in Xenopus laevis. Dev. Biol. 71, 323 - 340.

Davidson, E.H. (1976). Gene activity in early development. Academic press.

De Lorenzi, M., Rohrer, U. and Birnstiel, M.L. (1986). Analysis of a sea urchin gene cluster coding for the small nuclear U7 RNA, a rare RNA species implicated in the 3' editing of histone precursor mRNAs. Proc. Nat. Acad. Sci. U.S.A. 83, 3243 - 3247.

De Robertis, E.M. and Mertz, J.E. (1977). Coupled transcription - translation of DNA injected into Xenopus oocytes. Cell 12, 175 - 182.

Doenecke, D. and Tonjes, R. (1984). Conserved dyad symmetry structures at the 3' end of H5 histone genes. Analysis of the duck H5 gene. J. Mol. Biol. 178, 121 - 135.

Dolecki,G.J. and Smith,L.D. (1979). Poly (A+) RNA metabolism during oogenesis in*Xenopus laevis*. Dev. Biol. 69, 217 - 236.

Dumont, J.N. (1972). Oogenesis in *Xenopus laevis*. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136, 153 - 180.

Dworkin,M.B. and Dworkin - Rastl,E. (1985). Changes in RNA titers and polyadenylation during oogenesis and oocyte maturation in *Xenopus laevis*. Dev. Biol. 112, 451 - 457.

Edmonds,M. (1982). Poly (A) adding enzymes. The enzymes, Academic press, vol XV, 217 - 244.

Eliceiri,G. and Sayavedra,M. (1976). Small RNAs in the nucleus and cytoplasm of HeLa cells. Biophys. Biochem. Res. Comm. 72, 507 - 512.

Engel,J.D., Sugarman,B.J. and Dodgson,J.B. (1982). A chicken histone H3 gene contains intervening sequences. Nature 297, 434 - 436.

Fahrner,K., Yarger,J. and Hereford,L. (1980). Yeast histone mRNA is polyadenylated. N.A.R. 8, 5725 - 5737.

Falck - Pedersen,E., Logan,J., Shank,T. and Darnell,J.E. (1985). Transcription termination within the E1A gene of adenovirus induced by insertion of the mouse β - major globin terminator element. Cell 40, 897 - 905.

Fiers,W., Contreras,R. Haegeman,G., Rogiers,R., Van de Voode,A., Van Heuverswyn,H., Van Herreweghe,J., Volckhaert,G. and Ysebaert,M. (1978). Complete nucleotide sequence of SV40 DNA. Nature 273, 113 - 120.

Fitzgerald,M. and Shenk,T. (1981). The sequence 5' AAUAAA 3' forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell 24, 251 - 260.

Ford,J. and Hsu,M.T. (1978). Transcription pattern of *in vivo* labelled late simian virus 40 RNA: equimolar transcription beyond the mRNA 3' terminus. J. Virol. 28, 795 - 801.

Fradin,A., Jove,R., Hamamoto,C., Kaiser,H.D., Manley,J.L. and Prives,C. (1984). Splicing pathways of SV40 mRNAs in X.laevis oocytes differ in their requirements for snRNPs. Cell 37, 927 - 936.

Frayne,E.G., Leys,E.J., Crouse,G.F., Hook,A.G. and Kellems,R.E. (1984). Transcription of the mouse dihydrofolate reductase gene proceeds unabated through seven polyadenylation sites and terminates near a region of repeated DNA. Mol. Cell. Biol. 4, 2921 - 2924.

Galli,G., Hofstetter,H., Stunnenberg,H.G. and Birnstiel,M.L. (1983). Biochemical complementation with RNA in the Xenopus oocyte: A small RNA is required for the generation of 3' histone mRNA termini. Cell 34, 823 - 828.

Georgiev,O. and Birnstiel,M.L. (1985). The conserved CAAGAAAGA spacer sequence is an essential element for the formation of 3' termini of the sea urchin H3 histone mRNA by RNA processing. E.M.B.O.J. 4, 481 - 489.

Georgiev,O., Nous,J. and Birnstiel,M.L. (1984). Processing and nucleo - cytoplasmic transport of histone gene transcripts. Nucleic acids res. 12, 8539 - 8551.

Gick,O., Kramer,A., Keller,W. and Birnstiel,M.L. (1986). Generation of histone mRNA 3' ends by endonucleolytic cleavage of the pre - mRNA in a snRNP - dependant *in vitro* reaction. E.M.B.O.J. 5, 1319 - 1326.

Gil,A. and Proudfoot,N.J. (1984). A sequence downstream of AAUAAA is required for rabbit β globin mRNA 3' end formation. Nature 312, 473 - 474.

Golden,L., Schafer,U. and Rosbash,M. (1980). Accumulation of individual pA+ RNAs during oogenesis of Xenopus laevis. Cell 22, 835 - 844.

Green,M.R., Maniatis,T. and Melton,D.A. (1983). Human β globin pre - mRNA synthesised in vitro is accurately spliced in Xenopus oocyte nuclei. Cell 32, 681 - 694.

Greenberg,J.R. and Perry,R. (1972). Relative occurrence of polyadenylic acid in messenger and heterogeneous nuclear RNA of L - cells as determined by poly (U) hydroxylapatite chromatography. J. Mol. Biol. 72, 91 - 96.

Gurdon,J.B., Birnstiel,M.L. and Speight,V.A. (1969). The replication of purified DNA introduced into living egg cytoplasm. Biochim. Biophys. Acta 174, 614 - 628.

Gurdon,J.B., Lane,C.D., Woodland,H.R. and Marbaix,G. (1971). Use of frog eggs and oocytes for the study of mRNA and it's translation in living cells. Nature 233, 177 - 182.

Hagenbuchle,O., Wellauer,P.K., Cribbs,D.L. and Schibler,U. (1984). Termination of transcription in the mouse α amylase gene amy - 2a occurs at multiple sites downstream of the polyadenylation site. Cell 38, 737 - 744.

Hart,R.P., McDevitt,M.A., Ali,H. and Nevins,J.R. (1985) a. Definition of essential sequences and functional equivalence of elements downstream of the adenovirus E2A and early SV40 polyadenylation sites. Mol. Cell. Biol. 5, 2975 - 2983.

Hart,R.P., McDevitt,M.A. and Nevins,J.R. (1985) b. Poly (A) site cleavage in a HeLa nuclear extract is dependent on downstream sequences. Cell 43, 677 - 683.

Harvey,R.P., Whiting,J.A., Colem,L.S. Krieg,P.A. and Wells,J.R.E. (1983). An extremely variant histone H2a sequence expressed in the chicken embryo. Proc. Nat. Acad. Sci. U.S.A. 80, 2819 - 2823.

Hashimoto,C. and Steitz,J.A. (1984). U4 and U6 RNAs coexist in a single small nuclear ribonucleoprotein particle. N.A.R. 12, 3283 - 3294.

Hashimoto,C. and Steitz,J.A. (1986). A small nuclear ribonucleoprotein associates with the AAUAAA polyadenylation signal *in vitro*. Cell 45, 581 - 591.

Henikoff,S., Kelly,J.D. and Cohen,E.H. (1983). Transcription terminates in yeast distal to a control sequence. Cell 33, 607 - 614.

Henikoff,S. and Cohen,E.H. (1984). Sequences responsible for transcription termination on a gene segment in Saccharomyces cerevisiae. Mol. Cell. Biol. 4, 1515 - 1520.

Hentschel,C., Probst,E. and Birnstiel,M.L. (1980). Transcriptional fidelity of histone genes injected into Xenopus oocyte nuclei. Nature 288, 100 - 102.

Hentschel,C.C. and Birnstiel,M.L. (1981). The organisation and expression of histone gene families. Cell 25, 301 - 313.

Hernandez,N. (1985). Formation of the 3' end of U1 snRNA is directed by a conserved sequence located downstream of the coding region. E.M.B.O.J. 4, 1827 - 1837.

Hinterberger,M., Petterson,I. and Steitz,J.A. (1983). Isolation of small nuclear ribonucleoproteins containing u1, U2, U4, U5 and U6 RNAs. J. Biol. Chem. 258, 2604 - 2613.

Hofer,E. and Darnell,J.E. (1981). The primary transcription unit of the mouse ß - major globin gene. Cell 23,585 - 593.

Hofer,E., Hofer - Warbinek,R. and Darnell,J.E. (1982). Globin RNA transcription : A possible termination site and demonstration of transcriptional control correlated with altered chromatin structure. Cell 29, 887 - 893.

Konarska,M.M., Padgett,R.A. and Sharp,P.A. (1984). Recognition of cap structure in splicing *in vitro* of mRNA precursors. Cell 38, 731 - 736.

Konkel,D.A., Tilghman,S.M. and Leder,P. (1978). The sequence of the chromosomal mouse ß-globin major gene : homologies in capping, splicing and poly (A) sites. Cell 15, 1125 - 1132.

Kramer,A., Keller,W., Appel,B. and Luhrmann,R. (1984). The 5' terminus of the RNA moisty of U1 small nuclear ribonucleoprotein particles is required for the splicing of messenger RNA precursors. Cell 38, 299 - 307.

Krieg,P.A., Robins,A.J., D'Andrea,R. and Wells,J.R.E. (1983). The chicken H5 gene is unlinked to core and H1 histone genes. N.A.R. 11, 619 - 627.

Krieg,P.A. and Melton,D.A. (1984). Formation of the 3' end of histone mRNA by post - transcriptional processing. Nature 308, 203 - 206.

LeMaur,M.A., Galliot,B. and Gerlinger,P. (1984). Termination of the ovalbumin gene transcription. E.M.B.O. J. 3, 2779 - 2786.

Lerner,M.R., Boyle,J.A., Mount,S.M., Wolin,S.L. and Steitz,J.A. (1980). Are snRNPs involved in splicing? Nature 283, 220 - 224.

Lerner,M.R. and Steitz,J.A. (1981). Snurps and Scyrps. Cell 25, 298 - 300.

Madore,S.J., Wieben,E.D. and Pederson,T. (1984). Intracellular site of U1 small nuclear RNA processing and ribonucleoprotein assembly. J. Cell. Biol. 98, 188 - 192.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982). Molecular cloning A laboratory manual. Cold Spring Harbor Laboratory publications.

Manley,J.L. (1983). Accurate and specific polyadenylation of mRNA precursors in a soluble whole - cell lysate. Cell 33, 595 - 605.

Manley,J.L., Yu,H. and Ryner,L. (1985). RNA sequence containing hexanucleotide AAUAAA directs efficient mRNA polyadenylation in vitro. Mol. Cell. Biol. 5, 373 - 379.

Mason,P.J., Jones,M.B., Elkington,J.A. and Williams,J.G. (1985). Polyadenylation of the Xenopus β1 globin mRNA at a downstream minor site in the absence of the major site and utilization of an AAUACA polyadenylation signal. E.M.B.O. J. 4, 205 - 211.

Mason,P.J., Elkington,J.A., Lloyd,M.M. and Williams,J.G. (1986). Mutations downstream of the polyadenylation site of a Xenopus

ß globin mRNA affect the position but not the efficiency of 3' processing. Cell 46, 263 - 270.

Mattaj, I.W. and De Robertis, E.M. (1985). Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell 40, 111 - 118.

Maxam, A. and Gilbert, W. (1980). Sequencing end - labelled DNA with base specific chemical cleavages. Meth. Enzymol. 65, 499 - 560.

McDevitt, M.A., Imperiale, M.J., Ali, H. and Nevins, J.R. (1984). Requirement of a downstream sequence for generation of a poly (A) addition site. Cell 37, 993 - 999.

McLauchlan, J., Gaffney, D., Whitton, J.L. and Clements, J.B. (1985). The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for the efficent formation of mRNA 3' termini. N.A.R. 13, 1347 - 1365.

Melton, D.A, Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R.R. (1984).Efficient in vitro synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. N.A.R. 12, 7035 - 7056.

Mertz, J.E. and Gurdon, J.B. (1977). Purified DNAs are transcribed after microinjection into Xenopus oocytes. Proc. Nat. Acad. Sci. U.S.A. 74, 1502 - 1506.

Mimori, T., Hinterberger, M., Petterson, T. and Steitz, J.A. (1984). Autoantibodies to the U2 small nuclear ribonucleoprotein in a patient with scleroderma - polymyositis overlap syndrome. J. Biol. Chem. 259, 560 - 566.

Moore,C.L. and Sharp,P.A. (1984). Site - specific polyadenylation in a cell - free reaction. Cell 36, 581 - 591.

Moore,C.L. and Sharp,P.A. (1985). Accurate cleavage and polyadenylation of exogenous RNA substrate. Cell 41, 845 - 855.

Mount,S.M., Pettersson,I., Hinterberger,M., Karmas,A. and Steitz,J.A. (1983). The U1 small nuclear RNA - protein complex selectively binds a 5' splice site in vitro. Cell 33 509 - 518.

Murphy,J.T., Burgess,R.R., Dahlberg,J.E. and Lund,E. (1982). Transcription of a gene for a human U1 small nuclear RNA. Cell 29, 265 - 274.

Nevins,J.R. and Darnell,J.E. (1978). Steps in the processing of Ad2 mRNA: poly(A+) nuclear sequences are conserved and poly (A) addition precedes splicing. Cell 15, 1477 - 1493.

Nevins,J.R. (1983). The pathway of eukaryotic mRNA formation. Ann. Rev. Biochem. 52, 441 - 466.

Nevins,J.R., Blanchard,J.M. and Darnell,J.E. (1980). Transcription units of adenovirus type 2, termination of transcription beyond the poly (A) addition site in early regions 2 and 4. J. Mol. Biol. 144, 377 - 386.

Newport,J. and Kirschner,M. (1982)a. A major developmental transition in early Xenopus embryos: 1. Characterization and timing of cellular changes at the midblastula stage. Cell 30, 675 - 686.

Newport,J. and Kirschner,M. (1982)b. A major developmental transition in early Xenopus embryos: 11. Control of the onset of transcription. Cell 30, 687 - 696.

Newport, J.W. and Kirschner, M.W. (1984). Regulation of the cell cycle during early Xenopus development. Cell 37, 731 - 742.

Old, R.W., Woodland, H.R., Ballantine, J.E.M., Aldridge, T.C., Newton, C.A., Bains, W.A. and Turner, P.C. (1982). Organization and expression of cloned histone gene clusters from Xenopus laevis and Xenopus borealis. N.A.R. 10, 7561 - 7580.

Old, R.W. and Woodland, H.R. (1984). Histone genes: not so simple after all. Cell 38, 624 - 626.

Orkin, S.H., Cheng, T.C., Antonarakis, S.E. and Kazazian, S.E. (1985). Thalassemia due to a mutation in the cleavage - polyadenylation signal of the human β globin gene. E.M.B.O.J. 4, 453 - 456.

Patient, R.K., Harris, R., Walmsley, M.E. and Williams, J.G. (1983). The complete nucleotide sequence of the major adult β globin gene of Xenopus laevis. J. Biol. Chem. 258, 8521 - 8523.

Perricaudet, M., LeMoullec, J.M., Tiollais, P. and Pettersson, U. (1980). Structure of 2 adenovirus type 12 transforming polypeptides and their evolutionary implications. Nature 288, 174 - 176.

Pettersson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J.A. (1984). The structure of mammalian small nuclear ribonucleoproteins: identification of multiple protein components reactive with anti - U1 RNP and anti - Sm autoantibodies. J. Biol. Chem. 259, 5907 - 5914.

Price, D.H. and Parker, C.S. (1984). The 3' end of a Drosophila H3 mRNA is produced by a processing activity in vitro. Cell 38, 423 - 429.

Probst,E., Kressmann,A. and Birnstiel,M.L. (1979). Expression of sea urchin histone genes in the oocyte of Xenopus laevis. J. Mol. Biol. 135, 709 - 732.

Proudfoot,N.J. and Brownlee,G.G. (1974). Sequence at the 3' end of globin mRNA shows homology with immunoglobulin light chain mRNA. Nature 252, 359 - 362.

Proudfoot,N.J. and Brownlee,G.G. (1976). 3' non - coding region sequences in eukaryotic messenger RNA. Nature 263, 211 - 214.

Richter,J.D., Smith,L.D., Anderson,D.M. and Davidson,E.H. (1984). Interspersed poly (A) RNAs of amphibian oocytes are not translatable. J. Mol. Biol. 173, 227 - 241.

Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977). Labelling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237 - 251.

Rogers,J. and Wall,R. (1980). A mechanism for RNA splicing. Proc. Nat. Acad. Sci. U.S.A. 77, 1877 - 1879.

Rohrmann,G., Yuen,L. and Moss,B. (1986). Transcription of vaccinia virus early genes by enzymes isolated from vaccinia early virions terminates downstream of a regulatory sequence. Cell 46, 1029 - 1035.

Ruderman,J.V. and Pardue,M.L. (1978). A portion of all major classes of histone messenger RNA in amphibian oocytes is polyadenylated. J.Biol.Chem. 253, 2018 - 2025.

Sadofsky,M., Connelly,S., Manley,J.L. and Alwine,J.C. (1985). Identification of a sequence element on the 3' side of AAUAAA which is necessary for SV40 late mRNA 3' - end processing. Mol. Cell. Biol. 5, 2713 - 2719.

Sagata,N., Shiokawa,K. and Yamana,K. (1980). A study on the steady state population of poly (A)+ RNA during early development of Xenopus laevis. Dev. Biol. 77, 431 - 448.

Salditt - Georgieff,M. and Darnell,J.E. (1983). A precise termination site in the mouse β major globin transcription unit. Proc. Nat. Acad. Sci. U.S.A. 80, 4694 - 4698.

Sato,K., Ito,R., Baek,K. and Agarwal,K. (1986). A specific DNA sequence controls termination of transcription in the gastrin gene. Mol. Cell. Biol. 6, 1032 - 1043.

Schaufele,F., Gilmartin,G.M., Bannwarth,W. and Birnstiel,M.L. (1986). Compensatory mutations suggest that base - pairing with a small nuclear RNA is required to form the 3' end of H3 messenger RNA. Nature 323, 777,- 781.

Schroeder,T.E. and Strickland,D.L. (1974). Ionophore A23187, calcium and contractility in frog eggs. Exp. Cell Res. 83, 139 - 142.

Simmsen,C.C. and Levinson,A.D. (1983). Analysis of processing and polyadenylation signals of the hepatitis B virus surface antigen gene by using Simian virus 40 - hepatitis B virus chimeric plasmids. Mol.Cell.Biol. 3, 2250 - 2258.

Slater,I., Gillespie,D. and Slater,D.W. (1973). Cytoplasmic adenylation and processing of maternal mRNA. Proc.Nat.Acad.Sci.USA. 70, 406 - 411.

Slater,I. and Slater,D.W. (1974). Polyadenylation and transcription following fertilization. Proc. Nat. Acad.Sci.USA. 71, 1103 - 1107.

Strub,K., Galli,G., Busslinger,M. and Birnstiel,M.L. (1984). The cDNA sequences of the sea urchin U7 small nuclear RNA suggest specific contacts between histone mRNA precursor and U7 RNA during RNA processing. E.M.B.O. J. 3,2801 - 2807.

Strub,K. and Birnstiel,M.L. (1986). Genetic complementation in the Xenopus oocyte: co - expression of sea urchin histone and U7 RNAs restores 3' processing of H3 pre - mRNA in the oocyte. E.M.B.O. J. 5, 1675 - 1682.

Stunnenberg,H.G. and Birnstiel,M.L. (1982). Bioassay for components regulating eukaryotic gene expression: A chromosomal factor involved in the generation of histone mRNA 3' termini. Proc. Nat. Acad. Sci. USA 79, 6201 - 6204.

Thomas,P.S. (1980). Hybridisation of denatured RNA and small DNA fragments transfered to nitrocellulose. Proc. Nat. Acad. Sci. USA 77, 5201 - 5205.

Turner,P.C. and Woodland,H.R. (1982). H3 and H4 histone cDNA sequences from Xenopus: a sequence comparision of H4 genes. N.A.R. 10, 3769 - 3780.

Turner,P.C., Aldridge,T.C., Woodland,H.R. and Old,R.W. (1983). Nucleotide sequences of H1 histone genes from Xenopus laevis. A recently diverged pair of H1 genes and an unusual H1 pseudogene. N.A.R. 11, 4093 - 4107.

Twigg,A.J. and Sherratt,D. (1980). Trans - complementable copy - number mutants of plasmid COL E1. Nature 283, 216 - 218.

Van Dongen,W., Zoal,R., Moorman,A. and Destree,O. (1981). Quantitation of the accumulation of histone mRNA during oogenesis in Xenopus laevis. Dev. Biol. 86, 303 - 314.

Wickens,M.P. and Gurdon,J.B. (1983). Post transcriptional processing of simian virus 40 late transcripts in injected frog oocytes. J. Mol. Biol. 163, 1 - 26.

Wieben,E.D., Henninger,J.M. and Pederson,T. (1985). U2 small nuclear RNA precursors and their accurate 3' processing *in vitro* as ribonucleoprotein particles. J.Mol.Biol. 183, 69 - 78

Wigley,P.L., Sturm,R.A. and Wells,J.R.E. (1985). The tissue - specific chicken histone H5 gene is transcribed with fidelity in Xenopus laevis oocytes. J. Mol. Biol. 181, 449 - 452.

Wilson,A. (1986). Ph.D thesis university of Warwick.

Wilt,F.H. (1973). Polyadenylation of maternal RNA of sea urchin eggs after fertilisation. Proc.Nat.Acad.Sci.USA. 70, 2345 - 2349.

Wilt,F.H. (1977). The dynamics of maternal poly (A) - containing mRNA in fertilised sea urchin eggs. Cell 11, 673 - 681.

Woychik,R.P., Lyons,R.H., Post,L. and Rottman,F.M. (1984). Requirement for the 3' flanking region of the bovine growth hormone gene for accurate polyadenylation. Proc.Nat.Acad.Sci.USA 81, 3944 - 3948.

Yuo,C.Y., Ares,M. and Weiner,A.M. (1985). Sequences required for 3' end formation of human U2 small nuclear RNA. Cell 42, 193 - 202.

Zaret,K.S. and Sherman,F. (1982). DNA sequence required for efficent transcription termination in yeast. Cell 28, 563 - 573.

Zarkower,D., Stephenson,P., Sheets,M. and Wickens,M. (1986). The AAUAAA sequence is required both for cleavage and for

polyadenylation of simian virus 40 pre - mRNA *in vitro*. Mol. Cell. Biol. 6, 2317 - 2323.

Zeller,R., Nyffenegger,T. and De Robertis,E.M. (1983). Nucleocytoplasmic distribution of snRNPs and stockpiled snRNA - binding proteins during oogenesis and early development in Xenopus laevis. Cell 32, 425 - 434.

Zeller,R., Carri,M.T., Mattaj,I.W. and DeRobertis,E.M. (1984). Xenopus laevis U1 snRNA genes: characterisation of transcriptionally active genes reveals major and minor repeated gene families. E.M.B.O. J. 3, 1075 - 1081.

Zieve,G. and Penman,S. (1976). Small RNA species of the HeLa cell. Metabolism and subcellular localisation. Cell 8, 19 - 31.

Zieve,G.W. (1981). Two groups of small stable RNAs. Cell 25, 296 - 297.